University of Glasgow

THE ROLE OF GRANULOMA FORMATION IN IMMUNOLOGICAL ADJUVANCE

An analysis of the cellular composition of, germinal centre formation in, and antibody production by granulomata induced by Freund's adjuvants in the chicken.

by

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1. Henderson, D.C. and A.M. Smithyman. 1974. The simultaneous detection of two protein antigens in lymphoid tissues by combining immunofluorescence and autoradiography. J. Immunol. Methods. 6, 115

2. White, R.G., D.C. Henderson, and A.M.Smithyman. 1977. The role of Freund's complete adjuvant in the bird: An explanation of adjuvant action as a failure to switch off the normal antibody response. In: The VIIth International Symposium of Immunopathology, Bad Schachen, 1976. (Ed. P. Meischer). Schwabe, Basel. p. 361.

3. Smithyman, A.M. 1977. A combined autoradiography-immunofluorescence technique for the study of lymphocyte traffic in relation to antigen localisation. J. Immunol. Methods 17, 217.

4. Smithyman, A.M. 1977. A simple procedure for the isolation of germinal centres from chicken spleen. Dev. Comp. Immunol. 1, 263.

5. White, R.G. Organisation of the lymphoid tissues of Gallus domesticus. In: Differential diagnosis of Avian lymphoid leukosis and Marek's disease (Ed. L.N. Payne) Comm. of Eur. Comm. Luxemburg. 1976. p. 15.

"Fillet of a fenny snake,
In the cauldron boil and bake;
Eye of newt, and toe of frog
Wool of bat, and tongue of dog,
Adder's fork and blind-worm's sting,
Lizard's leg, and owlet's wing,
For a charm of powerful trouble,
Like a hell-broth boil and bubble."

Macbeth. Act IV, scene I.

Summary

The nature of the granulomata which are induced at the site of an injection of Freund's complete adjuvant, FCA (dead tubercle bacilli in mineral oil) containing an added antigen (HSA) were studied in the domestic fowl in relation to the adjuvant effect on antibody formation. The granulomatous lesions were induced in the pectoral muscle of the birds and examined at periods from one to twenty weeks and contrasted with granulomata resulting from injection of Freund's incomplete adjuvant, FIA, (antigen suspended in mineral oil). The cells forming these granulomata were studied in detail. As noted by authors working in other species (Spector and Lykke, 1966; Smith et al., 1970) the FCA granuloma is first dominated by emigration and proliferation of macrophages. This is followed by a gradual infiltration of lymphocytes, and plasma cells in an organised fashion so that the granuloma may come to resemble normal peripheral lymphoid tissue with "post-capillary venules", and primary lymphoid follicles and in the chicken occasional germinal centres and extensive antibody production, almost as if the granuloma had become a lymph node analogue.

One finding which has not been previously recorded was the extent to which germinal centres are involved in these processes. It was found that while local germinal centres were rarely if ever found in FCA granulomata beyond one week, FIA granulomata contained increasing numbers of large, well-defined germinal centres. This argued an important role for germinal centres in local sites. There seemed to be an inverse relationship between the number of germinal centres present and the level of circulating anti-HSA antibody observed.

Based on this observation the second part of this thesis examined the role of germinal centres in the immune response of the chicken, both in the spleen and in ectopic sites such as the granuloma. The results were tested against the hypothesis that germinal centres are involved in the feedback control of antibody biosynthesis, and the suggestion that the destruction or interference of germinal centres by the mycobacterial component of Freund's complete adjuvant is responsible for the abnormally high, late, or second phase, circulating antibody levels observed in FCA-treated animals.

It was concluded that the main adjuvant effect of FCA on the bird is a new and delayed biosynthesis of antibody (to HSA) derived from plasma cells and their precursors within the large epithelioid-cell granuloma which develops at the site of injection and that this late antibody production is dependent upon

a) stimulation by antigen emerging locally from the globules of water-in-emulsion in the granuloma at the injection site;

b) a supply of B cells to this granuloma from the earlier site of anti-HSA biosynthesis, namely the spleen;

c) an interference of the normal homeostatic mechanism of antibody production in the local granuloma by mycobacteria. The morphological result is that the germinal centre is prevented from developing in relation to the local granuloma; finally it is proposed that the interference mechanism could be based on the ability of a mycobacterial product to interfere with the ability of dendritic cells to take up antigen-antibody complexes.

INTRODUCTION

PREFACE

IMMUNOLOGICAL ADJUVANTS AND GRANULOMA FORMATION

One of the more interesting and important aspects of modern immunology is the study of adjuvants, a diverse group of substances which act to boost immunity. Their importance lies in one or more of the following areas: A) Their past and present use in world-wide veterinary and medical immunisation programmes, B) Their use as a laboratory tool in the production of highly specific anti-serum for therapeutic and research purposes, C) Their ability to induce certain experimental auto-immune diseases in animals, and D) Their present and potential use in the immunotherapy of cancer.

Closely related to adjuvant action and no less intriguing is the study of granuloma formation. The term "granuloma" is commonly used to describe the focal, chronic inflammatory lesions which are present in many pathological diseases caused by infectious (e.g. tuberculosis and leprosy) or non-infectious agents (e.g. berylliosis). The administration of adjuvants may also result in such lesions, the so-called adjuvant granulomata, either at the site of injection of the adjuvant material or at other sites throughout the body. Presumably such adjuvants mimic the action of naturally occurring granuloma-inducing irritants.

Neither the mechanisms of adjuvant action nor those of granuloma formation have been satisfactorily elucidated despite almost a century of investigation. The correlation noted above between adjuvant action and granuloma formation, suggests a possible approach to both these problems. The artificial production of granulomata by various adjuvant materials provides a suitable model for the detailed study of

/ both mechanisms. Thus if one could understand the basic principles of granuloma formation it might be possible to explain the mode of action of adjuvants and vice versa.

This thesis deals with such an approach: The formation and structure of granulomata resulting from local administration of emulsified water-in-oil adjuvants of the Freund type were studied in relation to the observed adjuvant effects. The results were tested against the hypothesis, first advanced by White (1973), that such adjuvants act by disrupting the normal feedback control mechanisms of the immune response, possibly by an effect on the formation of germinal centres.

The extreme diversity of adjuvant materials and the complex nature of granuloma formation makes this a difficult task. In order to analyse these related mechanisms it is essential, therefore, to consider separately the historical and contemporary background of each subject.

Immunological Adjuvants

The term immunological adjuvant (L. adjuvare:to help) is generally applied to substances which are incorporated into, or injected simultaneously with, an antigen, and potentiate non-specifically the ensuing immune responses (WHO report, 1976). In practical terms these adjuvants are added to vaccines to promote the greatest immunisation with the least antigen and number of doses given, an obvious logistic and economic asset in world health programmes.

In immunology adjuvants have been used in a number of ways, as summarised by White (1967a).

1) to convert apparently non-antigenic substances to obviously effective ones

2) to increase the level and duration of circulating antibody resulting from the antigenic stimulus

3) to lead to the induction of or increase in delayed hypersensitivity

4) to lead to the production of certain disease states such as thyroiditis, aspermatogenesis, "allergic" disseminated encephalomyelitis, adrenalitis or arthritis, and irredocyclitis ("adjuvant" disease)

Recent progress made in the understanding of disease, however, has tended to extend the emphasis of current use of adjuvants from simple prophylactic immunisation to wider spheres of activity. There are four major areas of this activity currently in progress involving use of adjuvants.

a) improvement and amplification of use of adjuvants in vaccines for world wide veterinary and human immunisation programmes

b) the production of large quantities of specific antiserum for research and diagnostic purposes (e.g. in immunoelectrophoresis or fluorescent antibody technique) or for therapeutic antisera

c) the induction of increased cell-mediated immunity against cells infected with intracellular agents (e.g. in leprosy) which are not overcome by natural immune defences

d) the induction of effective immune responses against tumour cells - generally referred to as immunotherapy of cancer

The last category of current research is responsible, perhaps more than the other three for the greatly increased interest in use of adjuvants in recent years. The possibility that adjuvants may help the host respond to its own tumour cells has generated much activity of late and there are at present many ongoing clinical trials of immunotherapeutic agents for use in the treatment of cancer (for comprehensive review see: Ann. N.Y. Acad. Sci., Vol. 277, 1976).

A list of the substances which exhibit adjuvant activity provide no evidence of a common chemical or biochemical structure to these agents. The list is rather bewildering in its diversity. Adjuvants may include items such as tapioca, bread, alum, mineral oil and peanut oil emulsions, acid-fast bacteria, exotoxins of Gram-positive bacteria, endotoxins of Gram-negative bacteria, silica, retinol, dextran sulphate, and polyanions. As suggested by White (1967(b) reading a list of adjuvant

substances is reminiscent of medieval alchemy. This strange group of substances has been assembled over the years on an empirical basis (Ramon, 1926 ; White, 1967a), with little regard for common scientific principles. The only common factor, indeed, is the lack of knowledge concerning the mechanism(s) of action of adjuvant materials. Consequently the diversity of adjuvants is matched only by the theories advanced to explain their unique properties.

The last decade has seen an improvement in this situation. This results in part from the increased understanding of the immune response and its component parts. Though adjuvant action is still not properly understood, the different cell populations affected by adjuvant materials are now becoming clearer and it is possible to construct more detailed hypotheses of the different mechanisms. It is also possible to distinguish between different types of adjuvants and they have been broadly grouped as follows:

1) Repository adjuvants, which include water-in-oil emulsions and aluminium compounds, e.g. Freund's complete and incomplete, alum.

2) Microbial adjuvants.

3) Parasitic adjuvants, e.g. Nippostrongylus brasiliensis

4) Natural and synthetic biochemical adjuvants.
   1. Fungal polysaccharides, e.g. lentinan
   2. Bacterial lipopolysaccharides, e.g. endotoxins
   3. Lysolecithin
   4. Lysosome labilisers

5. Polyanions, e.g. polyribonucleic acid

Repository Adjuvants

1.1. Aluminium and calcium compounds: These include aluminium phosphate, aluminium hydroxide, aluminium oxide and calcium phosphate, of which the first two are the most widely employed and longest used. These compounds have been extensively used in man and are considered safe though their use results in the formation of a small nodule or granuloma at the site of injection. Alum-precipitated antigens retain the antigen in high concentration locally at the site of injection and release it slowly. Increased antibody levels are thought to be a result of uptake of the antigen-bearing aluminium salt by macrophages with a consequent increase in the immunogenic effect beyond that of the same quantity of soluble antigen.

1.2. Emulsified water-in-oil adjuvants: These include probably the best-known adjuvants, the Freund's complete and incomplete adjuvant, and a more recent example, Adjuvant 65. Freund (1956) described two adjuvants: the incomplete adjuvant (FIA), which is a water-in-oil emulsion containing an emulsifying agent and paraffin oil, and the complete adjuvant (FCA) which contains killed and dried mycobacteria in addition. Both types of Freund's adjuvant are now widely used in immunology and experimental pathology laboratories, though the severe complications which accompany the use of the complete adjuvant have prevented its extensive use in man. The incomplete adjuvant has a good

safety record though the long term retention of mineral oil in the tissues is thought by some workers to be unacceptable. This has led to the development of the third type of water-in-oil emulsion, Adjuvant 65, which consists of an emulsion of aqueous vaccine in highly refined peanut oil using chemically pure emulsifying and stabilising agents (Hilleman, 1972). The major advantage of Adjuvant 65 lies in the fact that its components are readily metabolisable and have almost disappeared from the body within two months of injection. Follow up studies in man for 10 years after injection of Adjuvant 65 have shown no increase in clinically important adverse affects.

2. Bacterial adjuvants

A number of bacteria have gained recognition as adjuvants due to their immunostimulatory behaviour. These include the bacterial strains which are loosely grouped under the designation "Corynebacterium parvum", the Calmette-Guerin bacilli (BCG), and Bordetella pertussis.

2.1. C. parvum can be used as an adjuvant in three ways:

1) The bacteria may be included in a water-in-oil emulsion which results in an elevation of the serum antibody response.

2) They may be injected in high doses in saline intravenously with the subsequent intense activation and proliferation of macrophages in the recipient.

3) A small dose of bacteria, injected in combination with irradiated tumour cells or in the vicinity of a neoplasm

has been found to lead to the induction of specific T-cell immunity in the regional lymph nodes.

A granuloma develops at the site of injection of the bacteria either in saline or in oil. If injected intravenously miliary disseminated granuloma develop.

The use of C. parvum in the immunotherapy of human cancer patients is under intense investigation and a number of clinical trials are in progress (Israel, 1976; Hirshaut et al., 1976).

2.2. Calmette-Guerin bacillus (BCG)

BCG has achieved an important place as a non-specific immunostimulatory agent in human immunotherapy. The administration of BCG to mice, either locally or intravenously, results in an increased resistance to unrelated bacterial infections, enhances the clearance of particulate materials by the reticulo-endothelial system, enhances the production of antibodies, increases delayed-type hypersensitivity, accelerates the rejection of skin allografts and transplanted tumours, and inhibits carcinogenesis. Cell-mediated immunity is best stimulated by administration of freshly prepared cultures of BCG containing a high proportion of live bacilli. This has, in turn, led to a number of problems with allergic, immune-depressed human patients as in the production of multiple granulomata (Whittaker, 1974) following treatment with BCG. It also makes one wonder about the side effects of the BCG injection programme in schools. Do these result in immunostimulation?

2.3. Bordetella pertussis

The addition of killed B. pertussis bacteria to a variety of antigens prior to injection in rodents causes a marked increase in the ensuing antibody response and prolongs T- and B-cell memory. There is also a selective stimulation of the IgG and IgE antibody classes. The adjuvant mechanism of B. pertussis is not known though the bacteria are able to activate macrophages, induce a persistent lymphocytosis in the blood by preventing normal traffic of lymphocytes from the blood into lymphoid tissues through the post capillary venules of the lymph nodes (Morse and Barron, 1970).

Pertussis vaccine is often administered simultaneously with other vaccines such as tetanus toxoid and diptheria toxoid with a consequent increase in the antibody response to these agents.

3. Parasite adjuvants

Though not normally classified in the adjuvant category, infection by a nematode parasite, Nippostrongylus brasiliensis will substantially potentiate the reaginic antibody (IgE) response of rats previously injected with antigen and conventional adjuvant, for example, B. pertussis, (Orr and Blair, 1969), water-in-oil emulsions, and aluminium hydroxide (Jarrett , 1972). It seems therefore reasonable to include this parasite as an adjuvant though the parasite infection is ineffective if given at the same time as the antigen, the optimum administration time being 10 days after the antigen. Manipulations of

this kind are extremely important for the future understanding of the IgE system in rodents and in man, and have enabled the production of specific anti-IgE antisera for diagnostic and quantitative procedures.

4. Natural and synthetic biochemical adjuvants

4.1. Fungal polysaccharides, e.g. lentinan

An investigation of polysaccharides derived from fungi (Maeda et al., 1973) showed that lentinan, a 1-3-β-D glucose polymer was able to inhibit the growth of sarcoma 180 in intact but not thymectomised mice. The inference is that lentinan affects T lymphocyte helper function and the observation that the polymer is non-toxic to mice in large doses suggests that further research will prove productive.

4.2. Bacterial lipopolysaccharides (endotoxins)

The simultaneous administration of microgram quantities of lipopolysaccharides (from a variety of gram-negative bacteria) with antigens result in a greatly increased antibody response to the antigen. The adjuvant activity of the lipopolysaccharides is due to their lipid A component, which is common to them all. The lipid A molecule comprises two molecules of β (1→6) - linked glucosamine phosphate with myristic acid attached to the amino groups.

Unfortunately the lipopolysaccharides have a wide variety of biological activities which include pyrogenicity, plasminogen activation, adrenal cortex stimulation, interferon release and stimulation, and activation of the alternate complement pathway. Pure lipid A is pyrogenic in primates at a dose of 0.01 μg per Kg body weight. These factors make it unlikely that the lipopolysaccharides will be normally used as adjuvants.

4.3. Lysolecithin analogues

Synthetic analogues of lysolecithin have also been found to possess adjuvant activity (Sellin et al., 1974) and though these retain the surface-active properties of naturally derived lysolecithin, they are not readily metabolised via the normal phospholipid cycle and have an extended half-life in the body. Prior intraperitoneal or intravenous administration of such analogues as C_{18} ether hydroxylysolecithin and C_{16} ether deoxylysolecithin into mice may increase both the primary and secondary antibody responses to small amounts of antigen injected intraperitoneally 1 - 4 days later. The analogues have been shown to be potent activators of macrophages, and to facilitate intercellular bridging.

Lysolecithin analogues have also been shown to give substantial protection against a variety of tumour cells in mice and high doses of these analogues are non-toxic in guinea-pigs and baboons so they have great potential as a new class of adjuvants.

4.4. Lysosome labilisers

One common property shared by several adjuvant compounds is

the ability to cause disruption of cell lysosomes, with resultant leakage of the enzymes contained within. Examples of such substances are Vitamin A (Dresser, 1968), beryllium salts (Unanue, 1970) and toxic forms of silica (Wilkinson and White, 1966) and the non-toxic detergents. These compounds are thought to act through stimulation of macrophages.

4.5. Polyanions

Polyanions (Vogt et al., 1973), such as dextran sulphate and the polynucleotides polyadenylic-polyuridylic acid complex (poly A:U), and polyinosine-polycytidylic acid complex (poly I:C) have been shown to have striking effects on a wide variety of immune responses. In appropriate doses they can markedly enhance both humoral and cell-mediated immunity in normal animals and can restore the immune response of genetic low responders, neonatally thymectomised or aged animals (reviewed by Braun et al., 1971; Johnson et al., 1971). The polyanions are thought to act by replacing or stimulating T-cell function though they can enhance the response of macrophages as well (Johnson and Johnson, 1971). Polyanions have other biological activities, probably not related to adjuvant function, such as interferon induction, activation of C_3 complement component, and effect on blood clotting. In addition they have been found to potentiate autoimmune disease in NZB/W mice so it is unlikely that these adjuvants will find extensive use in man.

Freund's Complete and Incomplete Adjuvants

a) Historical Background

Perhaps the most powerful and widely used adjuvants in current experimental use are those water-in-oil emulsions named after Jules Freund (1956). There are two, Freund's incomplete adjuvant (FIA) which consists of an aqueous solution of antigen emulsified in mineral oil, and Freund's complete adjuvant (FCA), identical to above but with the addition of dried, heat-killed, mycobacteria in the oil phase. A wide variety of mycobacteria of human, bovine, atypical and saprophytic types have been shown to be effective in these adjuvant emulsions with an increase of both cell-mediated and humoral immunity (Freund and McDermott, 1942; Freund et al., 1948).

Though these adjuvants are most often associated with Freund the immunopotentiating effects of a mixture of mycobacteria and various oils has been recognised for many years. The first reference to this phenomenon is in a paper by Rabinowitch in 1897 in which he described the increased tuberculin sensitisation achieved by injecting tubercule bacilli in paraffin oil or paraffin wax. Two years later Grassberger (1899) described the enhanced cellular reaction of guinea pigs to an injection of mycobacteria in butter. Coulaud (1935), Saenz (1935) and eventually Freund and his co-workers (1937) succeeded in producing an intense and persistent sensitisation of rabbits with killed tubercule bacilli incorporated in paraffin.

On a slightly different track the experiments of Lewis and Loomis (1924, 1925) showed that the prior injection of living,

virulent tubercule bacilli into the peritoneal cavities of guinea pigs enhanced antibody formation to various antigens subsequently introduced by the same route. This was confirmed and extended by D nes and Schoenheit (1926) who showed that the injection of egg-white into a lesion caused by a large number of tubercule bacilli resulted in an enhancement of both cell-mediated and humoral immunity to that antigen. In their words "The tuberculous infection creates in an unknown manner the conditions necessary for the development of the local sensitiveness, as well as the conditions for an increased antibody production, which are not specific to a certain antigen". It was also noted that living tubercule bacilli were far more potent in enhancement than dead ones.

The crucial observation linking these two areas was made by Freund in 1937, and as is often the case in science, was the result of using the wrong group of animals at the right time! Freund's group observed accidentally that a group of guinea pigs injected with killed tubercule bacilli in paraffin oil for the purpose of sensitisation <u>also</u> produced antibodies to the bacilli in high titer, for at least 18 months after injection. Using emulsifying agents various antigens in aqueous solution or suspension were then emulsified in paraffin oil containing killed tubercule bacilli. For example guinea pigs receiving horse serum in this manner formed large quantities of precipitins to the horse serum proteins and gave an intense skin reaction to horse serum. Other antigens employed were bacteria, proteins, viruses, parasites and simple chemical compounds. In each case the paraffin oil alone enhanced and prolonged antibody

formation. If killed mycobacteria were present the adjuvant significantly increased delayed-type hypersensitivity and often further increased antibody production above the levels affected by the paraffin oil alone (Freund and Walter, 1944).

Another aspect to the use of these adjuvants soon become evident with the production of the experimental auto-immune disease, allergic encephalomyelitis by a single injection of brain tissue in FCA. (Freund, Stern, and Pisani, 1947). The disease was first produced experimentally in monkeys by Rivers and his associates in 1933 by giving 30 or more injections over a period of months to years.

Uses

It was obvious that here was a laboratory tool of great potential in biomedical research and the scientific community were not slow to take advantage of it. Both the complete and incomplete adjuvants are now widely used for the production of high levels of avid antibody against protein antigens, so much so that these adjuvants are available on a commercial basis (e.g.Difco Laboratories). The antibody produced is used in many ways, in forensic medicine, in diagnostic tests, for therapeutic purposes, in specific dyes (fluorescent antibody technique), in fact wherever use is made of the exquisite sensitivity of the antigen-antibody reaction.

In addition to allergic encephalomyelitis Freund's complete adjuvant has now been successful in the production of other experimental auto-immune diseases. These include thyroiditis (McMaster

et al., 1961), aspermatogenesis (Freund et al., 1953), adrenalitis (Colover and Glynn, 1958),nephritis (Frick, 1950) and arthritis and iridocyclitis (Pearson, 1956a,b).

A third use of Freunds adjuvants has been in the field of immunotherapy. The concept of utilising or enhancing the immune response to combat cancer is an old one (Coley, 1909) and efforts to achieve this using adjuvants have had mixed results (Hersh et al., 1973). For example, Graham and Graham in 1962 and Hughes and co-workers in 1970 treated cancer patients with FCA. Autologous tumour cells were emulsified with FCA and injected back into the patient. The results were complicated by the severe local and systemic side effects of the adjuvant.

Freund's incomplete adjuvant has been extensively tested in man in relation to influenza, poliomyelitis, adenovirus , and trachoma vaccines, and to a variety of allergenic preparations. It has also been applied to experimental rabies, canine distemper, infectious canine hepatitis, hog cholera, and bovine para influenza virus vaccines (Hilleman, 1966, 1967).

Effects in Mammals

Although the modes of action of Freund's adjuvants are incompletely understood the morphological and immunological responses to these complex materials are well documented in a number of animal species.

In general the subcutaneous administration of Freund's complete adjuvant results in the local accumulation and proliferation of

macrophages to form a large, painful, granuloma which may persist for many months. In addition there is an extensive proliferation of the reticulo-endothelial cells in the lymph nodes draining the area (Ehrich et al., 1947; Freund, 1947). Other findings include adjuvant induced polyarthritis, amyloidosis, nephrosis, and generalised granulomatous proliferation in various organs (Hilleman, 1967, Laufer et al., 1959).

When the complete adjuvant is injected intravenously there is a generalised proliferation of reticulo-endothelial, endothelial and fibroblast cells in many organs with the formation of multiple disseminated granulomata particularly in the spleen, liver, lungs, heart and lymph nodes (Rupp et al., 1960).

Subcutaneous administration of the incomplete adjuvant results in a small foreign body granuloma at the site of injection and the drainage of oil to local lymph nodes. However generalised granulomatous lesions have also been reported (Laufer et al., 1959).

Intravenous administration results in the same spread of the emulsion through the tissues but without the granulomatous response seen with the complete adjuvant. The morphological effects of the incomplete adjuvant in mice have been followed (Carter et al., 1966). The authors showed that the injection of 0.05 ml. of incomplete adjuvant into the hind foot-pad evoked an intense exudate of polymorphs and later pleomorphic mono-nuclear cells at the site of injection, many of which were loaded with fat. Similar changes occurred round oil droplets in the sinuses of draining lymph nodes and local lymphatic vessels.

When a protein antigen such as ovalbumin is added to the incomplete adjuvant mice responded to the antigen with a high, continuous antibody production which lasted for over a year (Herbert, 1968).

The effect of FCA in mice was followed by Humphrey and his colleagues (1967). The formation of a local granulomatous lesion at the site of a subcutaneous injection was accompanied by drainage of oil into local lymph nodes and progressive disorganisation of the lymphoid tissue.

Moore and his colleagues (1963) described the effects of an intravenous injection of the complete adjuvant (with or without added antigen) on rabbits. The production of widely disseminated granulomata in the spleen and lymph nodes was accompanied by a cellular proliferation typical of the primary response to a bland antigen but this was followed without interruption to the type of cellular response seen with a secondary response, i.e. the tissues did not return to normal by 6 - 8 days as is the case with a simple primary response. This response persisted for several weeks longer than with antigen alone. Antibody titres were higher and persisted much longer.

An important study of the effects of FCA was carried out by Humphrey (1963) in the rabbit. The use of FCA resulted in a large rise in serum gamma globulin, only part of which was specific for the incorporated mycobacteria. When an antigen was included in the FCA mixture about half of the 7S globulin was directed against it. Again this antibody production was associated with a local granuloma.

The effect of a subcutaneous injection of the complete adjuvant were studied by Spector and Lykke in the rat (1966). Use of FCA resulted in the formation of granulomata characterised by migration and proliferation of blood monocytes. In addition to these cells the lesions included lymphocytes, epithelioid cells, and giant cells.

The effects of FCA in the guinea pig have been studied by several groups. Freund and co-workers (1948) utilised FCA in the guinea pig to produce disseminated encephalomyelitis. Injection of homologous brain tissue in FCA resulted in extensive pathologic changes in the central nervous system, similar to those seen in demyelinating diseases. The guinea pigs became hypersensitive to brain tissue and produced antibodies to brain extract.

The immunological changes associated with an injection of FCA containing a protein antigen were followed in the guinea pig by Askonas and White (1956). They found that a single injection of ovalbumin-adjuvant mixture into the foot-pad resulted by 21 days in the formation of a large, firm, yellowish-grey, granulomatous mass at the site of injection. The homolateral, popliteal, inguinal, and lumbar lymph nodes were many times enlarged and their normal structure replaced almost totally by granulomatous tissue. The contralateral nodes were less affected and it is interesting to note that while the homolateral nodes contained few antibody-containing plasma cells the contralateral nodes showed an intense infiltration of specific antibody producing cells.

Wilkinson and White (1966) in the guinea pig correlated the size and appearance of the granuloma produced by FCA with the development

of delayed hypersensitivity and biosynthesis of γ_2 immunoglobulin.

The formation of a local granuloma caused by the subcutaneous injection of FCA was studied in sheep by Smith and his colleagues (1970). They cannulated the major lymphatic vessel draining the granuloma site and monitored the cell populations draining from the lesion. They noticed a significant rise in the flow of lymphocytes and concluded that these cells were being diverted from the blood to the granuloma. They also put forward the suggestion that the granuloma assumes the role of a splenic analogue with various cell types organised into different lymphoid compartments.

Finally Freund and his colleagues (1948) used FCA in rhesus monkeys to immunise them against malarial parasites. Monkeys receiving two injections of killed Plasmodium knowlesi with adjuvants survived when challenged with 1000 or more live parasites. Control animals immunised with killed parasites alone invariably died with high parasitemia after challenge.

Effects in Man

The effects of administration of Freund's incomplete adjuvant in man have been examined in great detail prior to the general use of the material (Hilleman, 1967). Details of the effects of the complete adjuvant are less readily available as this has been banned for use in man.

The early use of FIA in man was attended by severe local reactions such as nodule and cyst formation (Schofield, 1967). This was greatly

diminished by later stringent requirements governing the toxicity of the adjuvant components. A study involving use of FIA in 18,000 U.S. Army personnel showed no adverse long-term effects of the material. Neither is there any evidence for carcinogenesis caused by the mineral oil. However there are reports of development of delayed hypersensitivity in persons given allergens in incomplete adjuvant (Hilleman, 1967).

The complete adjuvant causes severe local and systemic abscesses in man. These abscesses are extremely painful and are often necrotic and for these reasons the use of FCA is not generally permitted in man. Consequently, details of the effects are harder to obtain. Exceptions to this are terminal cancer patients and there have been several reports of the effects and effectiveness of this material in immunotherapy (Hersh et al., 1973).

The dangers inherent in FCA are sharply illuminated by occasional reports in the literature of accidental injection of this material to laboratory workers. Berry and his colleagues (1975) described three cases of accidental injury with severe consequences and Chapel and August (1974) reported nine cases of varying severity. In six out of the nine cases a painful swelling developed at the site of injection which resolved slowly over many months. One patient suffered permanent damage to finger movement and other complications included a flu-like illness and persistent discharge from the lesions.

It seems likely that, in addition, the various pathological disorders caused by use of FCA in animals would apply to man. These

include adjuvant-induced polyarthritis, amyloidosis, mild nephrosis, and generalised granulomatous proliferation in various organs.

Extraction of the Active Component of Mycobacteria and Related Organisms

The obvious advantages and extraordinary effectiveness of Freund's complete adjuvant were tempered by the bizarre nature of the material. To the chemist and biochemist in the 1940's it was unthinkable that a "witches' brew" composed of whole bacteria mixed with emulsifiers and mineral oils could not be reduced to a simple, easily synthesised molecule. The witches' brew is still very much in evidence but chemists have made considerable progress in their task, so much that the simple, easily synthesised molecule may soon be widely available.

Early attempts to extract the active principle were based on the work of Freund and his co-workers (1948) who demonstrated that an alcohol-ether extract of mycobacteria added to a water-in-oil emulsion containing typhoid bacilli enhanced antibody formation to the bacilli. In the same year Raffel and Forney (1948) found that a chloroform, wax-like extract of Mycobacterium tuberculosis, Wax D, would potentiate the development of delayed-type hypersensitivity to picryl chloride and ovalbumin.

It was later found that only the wax D fractions from human strains and from an atypical strain, M. kansasii possessed such activity (White et al., 1964; White and Marshall, 1958). The

principal component of wax D is a peptidoglycolipid which is mixed with a lipopolysaccharide. The main chemical difference between the active and non-active waxes is the presence in the former of a peptide moiety. Hydrolysis of the active waxes yields a mixture of mycolic acids and a peptidoglycan. This latter contains the sugars, arabinose and galactose; the amino-sugars, N-glycolyl-muramic acid and M-acetyl glucosamine; and three amino acids, D- and C-alanine, D-glutamic acid, and meso-α, ε-diamino pimelic acid.

This work was followed by the extraction of water-soluble adjuvants (WSA) from the delipidated, trypsin and chymotrypsin treated cell walls of M. smegmatis by a procedure using lysozyme (Adam et al., 1972). A subsequent improvement of this technique produced another water-soluble adjuvant preparation called neo-WSA (Adam et al., 1973). These substances proved to be an arabino-galactan linked to a peptidoglycan. Both WSA and neo-WSA however, require to be administered as a water-in-oil emulsion to produce adjuvant effects. When given in this manner to guinea pigs it was shown that these adjuvant substances caused a local granuloma, increased serum antibody to an incorporated antigen, and induced a high level of cell mediated immunity to that antigen, in comparable fashion to whole bacilli (White et al., 1958).

Subsequently the neutral sugars were removed from the WSA peptidoglycan without affecting the adjuvant activity, (Adam et al., 1974), and more recently it has been demonstrated that synthetic N-acetyl-muramyl-L-alanyl-D-isoglutamine had the minimal structure required for adjuvant activity (Ellouz et al., 1974; Kotani

et al., 1975).

These results were obtained by immunisation of guinea pigs with the adjuvants and antigens in water-in-oil emulsions. Audibert and his colleagues (1976) then showed that the synthetic compound and another analogue N-acetyl-muramyl-L-alanyl-D-glutamic acid significantly augment the humoral immune response in mice when administered in aqueous solution.

Finally the same group has shown that the muramyl dipeptide is effective even when given orally, and the antigen given subcutaneously (Chedid et al., 1976).

It seems as if the 30 year search for the easily synthesised molecule is over; however, when tested in another animal system, in the adult fowl, the results were not as positive. In this system various human and bovine types of whole, heat-killed, mycobacteria, as well as M. avium and saprophytic strains, show a typical, prolonged adjuvant effect (French, Stark and White, 1970) and elicit a very large granulomatous reaction at the site of their injection in water-in-oil emulsion. Neo-WSA and synthetic muramyl dipeptide fail to show this adjuvant effect so it may be that future synthetic adjuvants may have to be tailor-made to resist the catabolic enzymic activity of individual species (White, 1976).

GRANULOMA FORMATION

2.1. Introduction

The formation of distinctive tissue masses or lesions known as granulomas or granulomata (singular: granuloma) is a crucial aspect of the pathogenesis of a wide range of diseases, affecting both animals and man. Some of these diseases are listed in Table 2.1. Two classic examples of granulomas are the tubercule of tuberculosis and the "gumma" of syphilis.

Granuloma formation is a well-known phenomenon in pathology, being first described in the early 19th century (Long, 1965) but no doubt recognised long before that, as there is evidence to suggest that diseases such as tuberculosis, leprosy, and schistosomiasis, which are characterised by granulomatous inflammation were endemic among early civilisations (for example, in the Dynasties of ancient Egypt, circa 3000 B.C. - Moodie,1923). Most pathology text books include detailed descriptions of the types of granuloma to be encountered, the types of cells found in these sites, and their effects on the tissues of the host.

It is suprising therefore to realise that until relatively recently, very little was known about the mechanisms which underlie granuloma formation. Thus one frequently encounters adjectives such as "enigmatic" or "mysterious" in the literature applied to granulomas and their effects. What was responsible for this lack of definition, and failure to understand the various processes which make up a granuloma?

There are three possible reasons. Firstly, the extreme complexity of the granulomatous reaction, and the many different types of cells

TABLE 2.1. AETIOLOGICAL CLASSIFICATION OF GRANULOMATA

Diseases characterised by granulomata of the following type :-

A. Infectious granulomata

Actinomycosis
Amoebiasis
Anthrax
Aspergillosis
Bagassosis
Blastomycosis
Coccidiomycosis
Filariasis
Glanders
Granuloma inguinale
Histoplasmosis
Interstitial pneumonitis
Leishmaniasis
Leprosy (tuberculoid)
Lymphogranuloma inguinale
Lymphogranuloma venereum
Rhinoscleroma
Schistosomiasis
Sporotrichosis
Syphilis
Torulosis
Tuberculosis

B. Foreign body granulomata

Adjuvant-induced granulomatosis
Asbestosis
Berylliosis
Pulmonary granulomatosis
Silicosis
Starch granulomatosis
Talc granulomatosis
Tattoo reactions
Zirconium granulomatosis

C. Granulomata of unknown aetiology

Allergic granulomatosis
Eosinophilic granulomatosis
Primary biliary cirrhosis
Sarcoidosis
(Hodgkin's disease)
(Mycosis fungoides)

involved has made it extremely difficult to understand the roles played by each in the reaction. Secondly, this complexity has been reflected in the literature in that there is lacking a unified nomenclature to the cells involved and the types of granuloma seen, with the obvious result of overall confusion. For example, there is still no commonly agreed definition of the term "granuloma". Thirdly, it was impossible to assign to the variety of cells available in a granuloma specific roles until these cells had been characterised at a general level. Thus the macrophages, monocytes, fibroblasts, eosinophils, lymphocytes, epithelioid cells, plasma cells and giant cells which go to make up a granuloma have had to await the growth of knowledge about their general biological roles before their presence in the granuloma became clear.

The rapid advance in immunobiology over the past twenty years, therefore, has been paralleled by an increased understanding of the biology of the granuloma. The separation of lymphocytes into the thymus-derived (T cell) and bursa-derived (B cell) systems and the vast increase in knowledge of the properties of these cells, and of the mononuclear phagocytes has significantly aided the elucidation of granuloma formation.

Granuloma Formation in Relation to Inflammation

Inflammation is a local tissue response to damage of any kind, whether caused by infectious, chemical, or physical agents, and has evolved from the primitive reactions to injury which are manifested by simple unicellular organisms. The response has probably evolved

as a means of limiting the spread of injury, eliminating the causative agent, and repairing the resulting damage to the tissues. The elegant studies of Metchnikoff (1892) laid the foundations for the modern understanding of inflammation, its causes, extent and effect in the protection of organisms.

Inflammation falls broadly into three categories; namely, acute, chronic, and chronic granulomatous inflammation. The first type, acute inflammation, involves the transient appearance of leukocytes from the general circulation, i.e. neutrophils, monocytes, and lymphoblasts, which then disappear on healing. Chronic inflammation is much the same though proliferation of the recruited monocytes and macrophages may occur and continue for long periods of time. The third type, chronic granulomatous inflammation results in the formation of long-lasting, distinctive, focal lesions, usually granular in nature and commonly called granulomata, formed mainly by the recruitment and proliferation of monocytes from the circulation. In addition they contain a variable complement of cells from the reticulo-endothelial system (RES), e.g. small lymphocytes, plasma cells, fibroblasts, macrophages, epithelioid cells and giant cells. It is this third type of inflammation which will be discussed here.

The Granuloma

The term "granuloma" (pl. granulomata) is essentially a misnomer. It means, literally, a tumour composed of granulation tissue. A granuloma is not a tumour however, and the term is usually used to describe pathological lesions which are the result of sub-acute or

chronic inflammatory changes associated with various protective and reparative processes. In addition, the lesions may produce considerable masses of new tissue which in some respects may come to resemble or simulate tumours (Fig.1.1.*a*.), hence the post-fix "-oma", denoting tumour. These so called granulation tissue "tumours" or granulomata may be divided into several groups according to the degree of complexity involved. These groups are to a certain extent, arbitrary, as there is disagreement between authors on the sub-division of granulomata. It is perhaps best to consider a selection of definitions and groupings at this point.

Definition

There is no universally agreed definition of the word "granuloma". Indeed a concise definition is difficult, if not impossible at the present time. The reason for this is simply that the study of granuloma formation has reached a point where the older morphological criteria are being increasingly challenged by new information and new terminologies.

Normally the term is applied to any chronic inflammatory reaction which is characterised by the accummulation and proliferation of mononuclear cells. Virchow (1863) described the granuloma as "a tumour or neoplasm made up of granulation tissue". It is defined in Webster's Dictionary (1966) as "a mass or nodule composed of chronically inflamed tissue marked by the formation of granulations and usually associated with an infective process". Herbert and Wilkinson (1971) refer to the granuloma as "a localised collection of macrophages that by their local accumulation and consequent mutual compression assume an appearance that

simulates a focus of epithelial cells. Hence these compressed macrophages are often called epithelioid cells". Epstein (1967) in a major review of granulomatous inflammation, defined the granuloma as "any focal chronic inflammatory reaction with a variable complement of cell types, necrosis, and stromal proliferation". Finally Adams (1976) gave a simpler description: "A granuloma is a compact, organised collection of mature mononuclear phagocytes, which is not necessarily accompanied by accessory features such as necrosis".

Classification

At the clinical level granulomata are usually divided into those of infectious, non-infectious (foreign body), and unknown origin. The diseases in Table 2.1. are listed in this fashion.

In addition to these examples, there are also other diseases with multiple lesions which present characters somewhat intermediate between those of granulomata and true tumours. Hodgkin's disease (Hodgkin's paragranuloma and granuloma) is one of these. Mycosis fungoides, which is characterised by the formation of multiple nodules of considerable size in the skin, is another. The nodules are at first of cellular character and a great vareity of cells are present:- lymphocytes, plasma cells, macrophages, multi-nucleate giant cells etc. - the lesions are of the granulomatous type but no organisms have been found and these diseases are considered to be neoplasms.

An additional group of lesions comes under the heading of experimental adjuvant granulomata and it is this category with which the present investigation deals. It has been found that a substantial and

varied group of compounds known as immunological adjuvants (L. adjuvare: to help) and used to boost or alter the immune response, when injected into tissues often produce a granuloma at the site of injection, e.g. Freund's complete adjuvant granuloma and alum granuloma. The formation and structure of such adjuvant granulomata closely follows that of their naturally induced counter-parts.

The infectious and foreign-body classification, based as it is on morphology, has been increasingly challenged of late. Other classification schemes have been proposed based on other criteria. Spector and Mariano (1975) have divided granulomas on the basis of cell turnover and have proposed a scheme involving two groups, the "high turnover" and "low turnover" lesions. Warren (1976) on the other hand, has separated granulomata according to their immunological component, into "immunologic" and non-immunologic". Allison and Davies (1975) have suggested a scheme dividing the granulomata according to their cellular components and level of complexity, into pure macrophage, macrophage/fibroblast, T cell/macrophage, B cell/macrophage, T/B/macrophage granuloma. Adams (1976) makes a distinction between a "pure granuloma" consisting of a compact, organised collection of mature mononuclear phagocytes and a "complex granuloma" which differs from the former in having accessory features such as necrosis.

The distinctions between types of granulomata proposed by some of these various schemes is not absolute. For instance in some atopic individuals, a "foreign-body", "low turnover", or "non-immunologic" granuloma may progress to the "high-turnover", "immunologic type" as happens in the case of sensitivity to beryllium and zirconium

(Epstein, 1967). Most individuals exposed to these agents react by forming small foreign-body nodules. A small percentage will develop delayed hypersensitivity to the metals and form large epithelioid (high turnover, immunologic) granulomas.

Alternatively the epithelioid, "high turnover", granulomas may regress and become low turnover foreign body reactions (Spector 1974 Adams 1976).

2.2. Aetiology of granuloma formation

The basic factor common to all granulomas seems to be the presence in the body of a focus of irritant or poorly digestible material. This material may be small enough to be ingested by phagocytic cells, as with tubercule bacilli or silica or too large as with schistosome eggs and Bentonite particles and plastic beads. The composition of this indigestible material then determines the type of granulomatous response which follows. Silica, for example, affects the lysosomes of macrophages ingesting it and this stimulates a powerful fibroblastic response; other materials are immunogenic causing the macrophage response to be associated with lymphocytic infiltration as in the case of Freund's complete adjuvant.

Another requisite for the development of the granuloma is persistence of the irritant material (Spector and Mariano, 1975). Organisms which are readily degradable by macrophages in culture cause only a transient acute inflammatory response in vivo. Organisms which resist degradation produce granulomas (Spector, Reichold and Ryan, 1970). An example of this type of persistent material is the cell wall of group A streptococci which has been found to persist in the rabbit for periods of up

to two years (Ginsburg and Sela, 1976).

There are a number of granulomatous conditions whose aetiology is unknown. The classical example of this group is sarcoidosis but also included are Crohn 's disease, eosinophilic granuloma and Wegener's granulomatasis. In addition the granulomata found in reputedly neoplastic diseases such as Hodgkin's disease or mycosis fungoides (granuloma fungoides) are also of unknown aetiology. Applying the broad principles outlined above one may postulate that these too, are caused by poorly biodegradable, antigenic, or infectious agents. Furthermore slight differences in the cellular components of the granulomata of these diseases may provide further clues as to the nature of such agents.

2.3. Kinetics of granuloma formation

A large tissue mass such as the granuloma poses considerable questions regarding the recruitment and origin of its variable complement of cell types (see above). One must consider how this mass of cells develops, is maintained, and finally disappears (for reviews see Epstein, 1967; Spector, 1969; Adams, 1976).

2.3.1. Formation, maintenance, and disappearance of granulomata

a) Macrophages

The classical component of the granuloma is the macrophage which is now also considered the precursor of the characteristic epithelioid and giant cells (Spector and Mariano, 1975). The work of Ebert and Florey in 1939 on the extravascular development of blood monocytes

suggested a logical origin for the granuloma macrophages and provided a basic framework for the studies of Spector and his colleagues over the past 10 years (see Spector, 1969; Spector and Ryan, 1970, for review). They demonstrated initially that the granuloma was built up by emigration of circulating monocytes from the blood (Spector and Lykke, 1966) in response to the granuloma-inducing irritant substance. In addition, the recently arrived blood monocytes appeared to be capable of rapid and extensive cell division. The granuloma appeared to be maintained both by immigration of cells from the blood and by cell division, (Ryan and Spector, 1969; Spector and Ryan, 1970).

The immigration kinetics were analysed by transfusing homologous, ^{3}H-thymidine-labelled monocytes into recipients with established granulomata. Using a polysaccharide seaweed extract, carrageenan, and M. tuberculosis in mineral oil the authors were able to establish clear-cut differences between the immigration and cell division of blood monocytes in the granulomata caused by these agents.

The carrageenan granuloma is characterised, after the first few days, by a low daily entry of between 10 - 20,000 monocytes. Cell division in this type of granuloma, after an initial high rate, falls to a persistent low mitotic index of 0.5 - 2.0%. This was referred to as a "low-turnover granuloma".

The mycobacterial granuloma, on the other hand, had a high daily entry of cells, about 250,000, which persisted for months. The mitotic index also remained high at a level of 4.6%. This type of lesion was designated a "high-turnover granuloma".

Thus two types of granuloma have been distinguished (Spector and

Mariano, 1975). High turnover granulomas are characterised by high levels of recruitment, division, death, and migration of cells, and are associated with irritants toxic to macrophages. Low turnover lesions are characterised by longevity of the macrophages with infrequent division and a low rate of recruitment and are associated with irritants of low toxicity, which are difficult to digest.

That granuloma formation is dependent on a supply of bone marrow cells has been demonstrated by a number of experiments in which the supply is cut off, usually by X-irradiation and the effects on the lesion studied. Thus if this is done to a mouse with a BCG granuloma the lesion is dramatically reduced in size within a week (Papadimitriou and Spector, 1972). Granulomata formed on glass cover slips in subcutaneous tissue show a considerable loss of macrophages if the bone marrow is destroyed (Ryan and Spector, 1970). Injection of M. tuberculosis in mineral oil into rats which had been previously whole body irradiated resulted in the complete suppression of the lesions normally seen at 3 and 7 days after such an injection (Spector and Willoughby, 1968). The granuloma could be restored by intravenous injections of bone marrow cells but not lymph node or thymic cells.

b) Fate of immigrant macrophages

Once they have gained entrance to the granulomatous lesion the macrophages have a number of possible fates (Spector and Mariano, 1975).

i) Death

Many of them die upon phagocytosing local toxic material and others are stimulated to divide as a result of their exposure to

mitogenic substances.

ii) Conversion to alternative form

There are three possible alternative forms: firstly, phagocytosis of non-toxic, indigestible material as in the case of low-turnover granulomas may convert the macrophages to a long-lived form.

Secondly, the macrophages may be converted to epithelioid cells either after immobilisation without phagocytic activity or immobilisation after successful phagocytic activity. The epithelioid cell is a characteristic feature of many types of granuloma e.g. in leprosy, tuberculosis and sarcoidosis. That they are of macrophage lineage has been proven in a number of ways. Avian macrophages, grown in tissue culture were observed to change into cells with epithelioid characteristics (Sutton and Weiss, 1966).

Granulomas in animals subjected to deprivation of macrophage precursors but fully supplied with lymphoid cells did not contain epithelioid cells (Spector and Willoughby, 1968). Cultivation of macrophages as monolayers on cellophane strips implanted in vivo resulted in the appearance of cells morphologically identical to epithelioid cells. The cultivated epithelioid cells were found to be poorly phagocytic as compared with the original macrophages but to be extremely active in pinocytosis and exocytosis. Their life-span is very short, usually not more than one week (Papadimitriou and Spector, 1971). The authors have proposed that the epithelioid cell represents a macrophage whose extra-cellular secretory powers have been enhanced at the expense of its phagocytic capacity.

Thirdly, the macrophages may fuse to form multinucleate giant cells,

known classically as foreign body giant cells or Langhans giant cells. Evidence for the macrophage origin of these cells again derived from isotopic labelling of macrophage precursors prior to their entry into established granulomata (Spector and Lykke, 1966; Ryan and Spector, 1970). The isotopic marker first appeared in macrophages then in multinucleate giant cells. Cultured avian macrophages also formed multinucleate giant cells (Sutton and Weiss, 1966). Detailed analysis of isotope labelling experiments and of electron micrographs taken during formation of multinucleate giant cells showed that these cells were formed by a process of fusion rather than nuclear division (Mariano and Spector, 1974). It appears that existing giant cells aquire new macrophages by fusion. The authors have suggested that this system may be one of surveillance against neoplasia as many of the incorporated macrophages exhibt unusual or abnormal chromosomal patterns.

iii) Emigration

Another possibility exists for macrophages in granulomatous lesions. They may migrate from the site of inflammation upon disappearance of the inflammatory stimulus (Spector and Mariano, 1975). Alternatively they may carry the irritant material with them as in guinea pigs given subcutaneous injections of alum, silica, or BCG vaccine (Gaafar and Turk, 1970) or in patients suffering from lepromatous leprosy, secondary syphilis, and sarcoidosis (Turk, 1973). The emigration of macrophages from granulomas and other inflamed sites has been the subject of an extensive review by Ginsburg and Sela (1976) in which they postulate that macrophages laden with irritant material migrate from the primary focus of inflammation to other tissue sites. The spread of

adjuvant arthritis in the rat (Pearson, 1956) and the dissemination of granulomata in rats (Waksman et al., 1960) and guinea pigs (Chase, 1959), treated with Freund's complete adjuvant also supports the idea of macrophage transport from the primary inflammation site.

c) Metabolic activity of macrophages in granulomata

The macrophages which enter granulomatous sites are at first in an immature, non-activated state. Once they have entered the lesion, in tuberculosis lesions for example, they develop a rich content of digestive enzymes and an ability to destroy ingested bacilli (Dannenberg, 1968). They are short-lived cells and many of them die after ingestion of toxic material. The released bacilli are then taken up by a fresh wave of macrophages, each of which ingests a smaller load, as the bacilli are shared between a greater number of cells. Eventually a series of new macrophages arises with the enzymic capacity to degrade the foreign substance or bacilli before they are themselves killed (Dannenberg et al., 1975). Ingestion and degradation of certain mycobacterial strains by macrophages is hindered by an apparent inability of the cell phagosomes to fuse with their lysosomes (Armstrong and D'Arcy Hart, 1971)

The biochemical and biological activities of mouse macrophages in the presence of known granuloma inducing irritants such as carrageenan, streptococcal cell walls, and antigen-antibody complexes has been investigated by Allison and Davies (1975). They have suggested that in contrast to macrophages in acute inflammatory reactions, those participating in sites of chronic inflammation differentiate and selectively synthesize a wide variety of biologically active compounds, including many enzymes.

A recent investigation by Unanue (1976) concluded that macrophages have an important secretory function and may release three sets of materials into their surroundings: a) enzymes that affect extracellular proteins, such as elastase, collagenase, plasminogen activators, and lysosomal proteinases; b) materials involved in defence reactions - complement proteins, lysozyme, interferon ; and c) materials that affect the behaviour of other cells - an inhibitor of DNA synthesis, a mitogenic factor, a lymphocyte maturation factor, and a factor enhancing antibody formation. It seems likely that some or all of these products are operative in granulomatous lesions.

2.3.2. Role of lymphocytes in granuloma formation

The numerical dominance of macrophages in granulomatous lesions (80% of the cells in Freund's complete adjuvant granulomata) at first tended to obscure the roles of the other cells present, notably lymphocytes, but also eosinophils and fibroblasts. It has long been known that in certain types of granuloma, maturation is accompanied by increased colonisation of the lesion by lymphocytes presumably migrating from the bloodstream. (White et al., 1955; Suter and White, 1954; Spector and Lykke, 1966; Smith et al., 1970; French et al., 1970). The significance of their presence was not always fully appreciated. The well-known tuberculosis expert, Arnold Rich (1951) once described the lymphocytes in a tuberculous granuloma as "phlegmatic spectators watching the turbulent activities of the phagocytes".

Significant advances have been made recently concerning the part played by lymphocytes in granuloma formation, but the full extent of their involvement has yet to be determined. The number of lymphocytes in,

or passing through the granuloma may be very large, as evidenced by experiments involving the study of granulomata induced in the hind limbs of sheep by injection of Freund's complete adjuvant (Smith et al., 1970). The major lymphatic vessel draining the granulomatous site was cannulated and the cells in the draining lymph identified and counted. The initial period after injection was dominated by polymorphonuclear neutrophils but after 48 hours the lymphocyte output increased until between 1 and 4 weeks the number of lymphocytes coming from the granuloma was about the same as a normal lymph node. The entry point for these lymphocytes was considered to be through reactive blood vessels structurally similar to lymph node post-capillary venules, the normal site of recirculation of lymphocytes from blood to lymph (Gowans and Knight, 1964). It is also known that the inflammation induced by living tubercule bacilli is accompanied by changes in local small blood vessels. Within several hours, endothelial cells lining these vessels hypertrophy and become increasingly pyroninophilic (Laporte, 1934). Small lymphocytes are often found in the walls of these reactive vessels, indicating lymphocyte traffic across the vessels.

The origins of these lymphocytes have been hinted at in the literature. Thus in some situations the immigrant lymphocytes have been identified as T-cells (Ward et al., 1970; Asherson, 1973; McGregor and Logie, 1975) while in others as B-cells (Allison and Davies, 1973; Loewi and Papamichael, 1973; McCluskey and Leber, 1974).

i) Thymus dependence of granuloma formation

Despite the correlation of delayed hypersensitivity with granuloma formation which occurrs in tuberculosis, cell-mediated immunity

was not originally thought to be involved in this process. The tubercule was thought to result from the irritant effects of bacillary lipids (Rich, 1951). Warren and his colleagues (1967) however, investigated the role of delayed hypersensitivity in granulomatous inflammation more closely, using as an experimental model the schistosome egg granuloma (Von Lichtenberg, 1962). They showed that the granuloma which forms round schistosome eggs in the lungs of mice resulted from delayed hypersensitivity reactions to soluble egg antigens. The granulomatous reaction could be transferred by cells but not serum, and could be suppressed by agents which inhibited cell-mediated reactions but not by inhibitors of antibody formation (Warren and Boros, 1975). Based on this study a technique was developed for the study of granulomatous inflammation using small bentonite particles with adsorbed soluble antigens (Boros and Warren, 1973). Use of soluble antigens derived from M. tuberculosis and Histoplasma capsulatum resulted in the formation of small foreign body granulomas. In contrast sensitised mice responded to the bentonite particles with large granulomas containing lymphocytes, macrophages, eosinophils and epithelioid cells. The authors have suggested that granuloma formation is mediated by a release of lymphokines from memory T cells upon exposure to antigen. They have also advanced the suggestion that granulomas should be divided into immunologic and non-immunologic categories. The former result from an immunological memory response, the latter do not.

These studies were confirmed using a system in which various protein antigens were covalently coupled to large polyacrylamide beads and injected into guinea pigs (Unanue and Benacerraf, 1973; Unanue, 1975).

The same cell-mediated criteria held for these experiments as in those described before. Immune reactivity could be conferred to normal guinea pigs by transfer of lymphocytes but not by injection of serum antibodies.

Other evidence for the contribution of delayed hypersensitivity to granuloma formation comes from the studies of Dannenberg and his co-workers (1975) who showed that BCG lesions of reinfection in rabbits enlarged more rapidly than primary lesions. The rabbits had been vaccinated with BCG 3 to 4 weeks previously and were therefore tuberculin positive before the new lesions were begun.

Further evidence for a thymic role in granulomatous reactions comes from experiments in which animals, after thymectomy, are no longer able to form a granuloma. For example, thymectomised mice no longer respond to an infection with Mycobacterium leprae (Rees et al., 1967) and neonatally thymectomised chickens fail to produce a typical large granuloma at the site of injection of Freund's complete adjuvant (White, 1971).

Furthermore it has been demonstrated recently (Wynne et al., 1975) that a mitogenic factor present in inflammatory exudates could stimulate macrophages to divide in vitro. In the same year it was shown that a factor produced by guinea pig lymph node lymphocytes under conditions of intense delayed hypersensitivity was also capable of inducing guinea pig macrophages to proliferate in vitro (Hadden et al., 1975). The authors suggested that the factor, called macrophage mitogenic factor (MMF) was of T cell derivation.

ii) Role of B cells in granuloma formation

The evidence for B cell participation in granuloma formation is not so substantial. A form of circumstantial evidence has been available for many years in that it is well-known that granulomata formed at the site of injection of adjuvants may produce large quantities of antibody (Freund et al., 1952; Askonas and Humphrey, 1958; French et al., 1970). In conjunction with the presence of large numbers of plasma cells this suggests that the B cell precursors of antibody producing plasma cells are available in the lesion. In the synovia of patients with rheumatoid arthritis many B cells are present and immune complexes are demonstrable (Allison and Davies, 1975; Loewi and Papamichael, 1973). Immune complexes are known to be a potent stimulus of granuloma formation (Spector and Heesom, 1969; Allison, 1975; Warren, 1976) and it is reasonable to assume that B cells participate in this process.

2.3.3. Other cell types

Other cell types involved in granulomatous inflammation include fibroblasts (seen, for example in tuberculoid granulomas or in lesions induced by silica) and eosinophils (in eosinophilic granulomas, tuberculoid granulomas and in those caused by Schistosoma japonicum. Little is known about the contribution of the latter cells but it is thought (Warren, 1976) that their presence is indicative of high local concentrations of antigen-antibody complexes.

Summary

The granuloma, then may be envisaged, in simplified terms as a compact, highly organised collection or framework of macrophages

existing in a state of dynamic equilibrium. This equilibrium is maintained both by immigration of fresh monocytes from the blood and by division of the macrophages in situ.

Superimposed upon this basic macrophage framework may be a number of different cell types such as fibroblasts, T and B cells, plasma cells and eosinophils whose presence is determined by the nature of the original irritant substance.

If the irritant is inert, e.g. carbon, there is a short lived proliferation of macrophages which ceases when the carbon particles have been ingested and removed.

At the next level of complexity the irritant material is not inert but intereacts with the macrophage as in the case of silica and asbestos. The result is a powerful macrophage and fibroblast response. If the irritant material is immunogenic then the reaction is more complex still. In addition to monocytes, T cells or B cells or both migrate into the reactive site and release factors which result in the establishment of an extensive lymphoid component in the granuloma, so much so that the granuloma may occasionally assume characteristics of both the spleen and lymph nodes such as plasma cell differentiation and antibody production.

While it is possible that certain granulomas are purely T cell/ macrophage or B cell/ macrophage lesions it is probable that most naturally occurring granulomata include a variable combination of the cell types discussed above.

THE PRESENT PROJECT

The present project was based on an observation, made some years ago, that the injection of Freund's complete adjuvant into the breast muscle of a chicken (Gallus domesticus) resulted in the formation of a large and striking local granuloma (White and Marshall, 1964). The development of the granuloma was accompanied by an increase in delayed hypersensitivity to tuberculin.

When a protein antigen such as human serum albumin (HSA) was included in the aqueous phase of the adjuvant emulsion no increase in the peak primary serum antibody response was seen as compared with control birds injected with antigen in saline (Steinberg et al., 1970). Neither was any effect noticed on the secondary response when a second injection of HSA was given 56 days later.

However the serum antibody levels of the adjuvant-treated birds did not show the characteristic fall to low levels at 18 days after injection, normally seen in chickens during a primary immune response (Ivanyi et al., 1966). Instead an initial fall from the peak of the primary response was interrupted by a second, slow rise in antibody production beginning at about the 21st day and reaching a peak somewhere between the 42nd and 59th days. This second peak reached levels 10 - 100 times those of the primary peak.

An investigation of various tissue extracts of these birds revealed the presence of large quantities of antibody in the local granuloma whereas the spleen, caecal tonsils, liver, bone marrow and lungs showed very little (French, Stark and White, 1970). In this

the chicken is similar to the rabbit and the horse, but not the guinea pig. In the two former species the local granuloma found at the site of injection of Freund's complete adjuvant makes a major contribution to the overall antibody production (Askonas and Humphrey, 1958).

Further investigation of this phenomenon (White, 1971) explored the effects of bursectomy and thymectomy. Bursectomy plus irradiation had a dramatic effect on both phases of the antibody response to HSA in FCA and resulted in the complete elimination of both responses. After thymectomy plus irradiation the antibody response from 10 days onward, was considerably reduced, down to levels similar to that of birds receiving this antigen in FIA.

In the bursectomised birds the granuloma at the site of injection was normal in size and appearance though histological examination showed an absence of plasma cells. In thymectomised birds, however, the granuloma was considerably decreased in size so that in some birds it was difficult to find. Histological sections showed a great decrease in the number of epithelioid and giant cells.

These facts were interpreted by White to mean that the second phase of antibody production to HSA in FCA injected birds was thymus-dependent. The mycobacteria in the FCA attracted a population of thymus-dependent cells to the granuloma and these cells, in co-operation with bursa-derived cells accounted for the major part of the second antibody peak.

It is necessary at this point to return to a consideration of the normal kinetics of an intravenously injected protein antigen in

chicken. These have been analysed in great detail (White, French and Stark, 1970). It was shown that the biosynthesis of antibody by plasmacytes in the red pulp of the spleen (as judged by the fluorescent antibody technique) starts within 24 hours of intravenous administration of antigen and rises sharply to reach a maximum at day 6, followed by a sudden reduction in numbers so that few antibody containing plasma cells could be seen in the red pulp by day 8. This histological sequence slightly preceded the serum antibody levels which were seen to rise from the fourth day to reach a maximum at the seventh to ninth days, then rapidly declined to very low levels by the 18th day. The exponential characteristics of this decline in antibody production were matched exactly by a passively administered I^{131} trace-labelled sample of chicken 7S gamma globulin injected intravenously into a bird on the third day after an injection of HSA (White, 1973).

The rapid disappearance of antibody-producing plasma cells from the spleen after day 6 and the rapid decline in serum antibody levels occurring after 9 days suggested a sharp switch-off of antibody production operated by a feedback mechanism. Further confirmation of this type of mechanism came from the demonstration that passive antibody administered at the same time as antigen caused a severe inhibition of antibody formation in the host animal (White, 1973).

In order to prevent further antibody production one must envisage a scheme whereby the B cell precursors of antibody-forming cells are either removed from the influence of antigen or suppressed in some way.

At the same time that the antibody is acting to produce the negative feedback there is no free antigen in the circulation (White et al., 1970). Most of the antigen has been catabolised except for a small fraction present in the form of antigen-antibody complexes at the surface of "dendritic cells" in the germinal centres in the white pulp of the spleen.

Neither are there any B cell precursors of antibody-forming cells available in the circulation at this time. Chickens injected with HSA become refractory to further induction of antibody in the period immediately following the primary response - from day 7 to 16. At this same time it was found that the number of rosette-forming cells (B cells) in the blood and spleen were considerably diminished (White, 1973). The question which immediately presents itself is where are these cells?

To return to the effects of protein antigen administration in the chicken; one of the most obvious results of immunisation of this kind is the formation in the spleen of large numbers of germinal centres (White, 1963; White et al., 1970). These structures form at about the fourth day after antigen administration, are seen at first as small collections of antigen-bearing dendritic cells which enlarge considerably from the fourth to the seventh days by the progressive inclusion of numerous small lymphocytes. White (1963) suggested that these small cells were in fact B cells and that the germinal centres were formed by the segregation of B cells passing through the spleen by the antigen-antibody complexes on the dendritic cell surfaces. Further evidence to support this came from the demonstration (Durkin

et al., 1972; Eslami et al-see De Sousa, 1973) that bursa cells radioactively labelled in vitro and injected into recipients localise to germinal centres within 24 hours. It was thought originally that germinal centre formation was concerned with antibody production but the kinetics did not support such a premise. Firstly, antibody-producing plasma cells are seen in the spleen between 48 and 72 hours before antigen-containing germinal centres; secondly, at the height of the germinal centre response few plasma cells are still visible in the spleen; thirdly, except under exceptional circumstances antibody-containing cells are rarely seen in germinal centres.

These observations led White (1973) to propose a radically new hypothesis of feedback control. This scheme proposed that germinal centres were ideally suited, by virtue of their dependence on antibody, their antigen-containing cells and segregation of B cells to play an important role in antibody homeostasis. As the centres form they remove potential antibody-forming cell precursors from the circulation and "freeze" them temporarily in a separate lymphoid compartment, separated from any further similar antigenic stimulation.

When the theory was applied to the action of Freund's complete adjuvant, White (1973) has proposed that the adjuvant in some way interferes with the homeostasis of antibody production, possibly by disrupting or preventing germinal centre formation, thus releasing increased numbers of potential antibody-forming cell precursors into the general circulation.

The main aim of the present project was to explore the various aspects of this hypothesis in greater detail. There seemed to be a fundamental relationship between the formation of a local granuloma at the site of an adjuvant injection, the observed high levels of antibody which result from that injection, and the kinetics of germinal centre formation. A suitable working hypothesis suggested that an expanded population of B cell antibody-forming cell precursors was being diverted toward the granuloma and there encountering conditions (such as antigen, T cells and macrophages) conducive to extensive and prolonged antibody production.

A number of specific questions required answers:

1) What are the histological events taking place in the local granuloma and how do these influence subsequent events?

2) How critical are the conditions governing the use of the adjuvant emulsion, for example, what effect has the age of the birds, the dose of bacilli, the omission or separation of the various components of the injection mixtures, on the subsequent adjuvant response?

3) What is the role of germinal centres under the conditions effected by adjuvant emulsions?

4) What is the nature of the triangular relationship existing between the adjuvant, the granuloma, and germinal centre formation?

MATERIALS

MATERIALS

Adjuvants:

Freund's incomplete adjuvant (FIA, a water-in-oil emulsion consisting of antigen solution, the mineral oil, Drakeol 6VR and the emulsifying agent Arlacel A) and Freund's complete adjuvant (FCA, as above but with the addition of lyophilised, heat-killed Mycobacterium avium) were prepared in the laboratory as described in the methods section.

Animals:

Chickens

The experimental work to be described was carried out in domestic fowl - Gallus domesticus.

Both male and female birds ranging from five week to 18 week old animals were used. White Leghorn (Thornber 808) and crossed White Leghorn/Rhode Island Red randomly bred stains were used. They were fed for the first six weeks on baby chick crumbs and thereafter on 50 per cent intensive growers and 50 per cent intensive layers pellets obtained from the British Oil and Cake Mills (Glasgow Branch). The newly hatched chickens were reared in Chicken Brooder Units (North Kent Plastic Cages Ltd.) and supplied with electric heaters for warmth during the first two weeks of life. Older birds were housed, in groups of 6 - 8 birds, in cat cages. Drinking water was supplied ad. lib. Animals that received injections of radio-iodine labelled proteins had their drinking water supplemented with 0.01% potassium iodide and 0.075% sodium chloride.

Rabbits

New Zealand white rabbits, obtained from Kingford Conies, Grey Tey, Colchester, England, were used to raise high-titre antisera.

Sheep

Specific antisera were also raised in randomly bred, Scottish Black-face sheep which were reared and housed on the University of Glasgow Farm at Cochno, Hardgate, Dunbartonshire.

Arlacel A:

Arlacel A (Mannide Mono-oleate) is an emulsifying agent manufactured by the Atlas Powder Co., Wilmington, Delaware, U.S.A. A supply of Arlacel A was obtained from Evans Medical Ltd., Speke, Liverpool.

Distilled Water:

Glass distilled water was used.

Drakeol 6 VR:

Drakeol 6 VR was most kindly supplied as a gift to the Department by the Pennsylvania Refining Company, Butler, Pennsylvania, U.S.A.

Iodine ^{131} or ^{125}, ^{3}H-adenosine, and ^{3}H-uridine:

These isotopes were obtained from the Radiochemical Centre, Amersham, England. They were dispensed in mCi amounts by the Western Infirmary Isotope Department.

Mycobacterium Avium (Strain D4 ER):

This preparation was kindly supplied by the Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey, England. They were supplied in a heat-killed and freeze-dried form.

Stains:

Harris's haematoxylin and eosin, Unna-Pappenheim, auramine, rhodamine, acridine orange and carbol thionin were obtained from G.T. Gurr Ltd., London, England.

Syringes and Needles:

Sterile, plastic, disposable, syringes, sizes 1, 2, 5, 10 and 20ml were used in these studies. They were obtained from Becton, Dickinson and Co. Ltd., Drogheda, Ireland. They were used once and destroyed. Gillette Sc imitar sterile disposable hypodermic needles, of different sizes according to size of animals and purpose, were also used. They were obtained from Gillette Surgical, Isleworth, Middlesex, England.

Tubes for Storing Sera:

The tubes used were disposable conical FS auto-analyser cups with polythene caps, obtained from Sterilin, Richmond, Surrey, England.

METHODS

1. PREPARATION OF MATERIALS FOR INJECTION

Human Serum Albumin

The human serum albumin (HSA) used in these experiments was obtained in a purified crystalline state ("Reinst") from Behringwerke, Marburg, Germany.

Human Gamma-Globulin

Human gamma-globulin (HGG) was kindly presented by Dr. John Wallace, Blood Transfusion Service, Law Hospital.

Preparation of Monomeric HGG

A solution of 20 mg/ml of HGG in 0.01M phosphate - 0.65M sodium chloride (PBS pH 7.2) was centrifuged at 140,000g for 90 minutes in a Beckman preparative ultracentrifuge. The upper two thirds of the fluid was removed. Sodium sulphate solution (2.18M) was added dropwise until a final molarity of 0.62M was reached. After stirring for 30 minutes the solution was centrifuged at 500g for 15 minutes and the supernatant dialysed against PBS until free from sulphate ions. This product comprises HGG monomer. The protein content was measured at 280nm wave length in a Unicam SP800 spectro photometer. The method follows that described by Brown, Schwab and Holborrow (1970).

Adjuvant Mixtures

Simple Water-in-Oil Emulsions or Incomplete Freund's Adjuvant (FIA)

Water-in-oil (W/O) emulsions containing antigen were prepared from the following reagents: antigen solution in 0.15M

/ saline; mineral oil (Drakeol 6VR) and emulsifying agent, Arlacel A. Three volumes of Drakeol 6VR were thoroughly mixed with one volume of Arlacel A in a bijou bottle large enough to just contain 5 volumes, and 1 glass bead (5mm diam.) was added. The antigen was dissolved in the volume of 0.15M saline. One third of the antigen solution was taken up in a disposable hypodermic syringe and forcibly injected into the oil mixture through a 0.5mm needle. The container was then capped and shaken vigorously before the next portion of antigen solution was added in the same way. After the final addition of antigen the mixed emulsion was taken up into the syringe through a wide bore (1mm) needle and squirted out again through the fine bore 0.5mm needle. The container was capped and shaken vigorously on a mechanical shaker (Flask shaker, Griffin and George Ltd., England) for at least 30 minutes, to ensure that the water phase was finely dispersed throughout the oil phase. Before use all water-in-oil emulsions were tested by allowing a portion to drop into cold tap water. If they were true water-in-oil emulsions they remained as discrete drops on the surface of the water and did not disperse. Emulsions were used within a day of preparation.

Freund's Complete Adjuvant (FCA)

Freund's complete adjuvant consists of a suspension of mycobacteria in the oil phase of a water-in-oil emulsion. In the experiments on birds, Mycobacterium avium (Weybridge, heat-killed) at a dose of 5mg per animal unless otherwise stated was used. In the production of antisera in rabbits and sheep 1 - 2mg of M. tuberculosis (Weybridge, Strain C, heat-killed) was used. The

/ mycobacteria were weighed out into a sterile, dry, glass container and finely ground with a sterile glass bead (5mm diam.) on a mechanical shaker for 10 minutes. The oil mixture was added and the shaking procedure continued for a further 10 minutes to ensure an even distribution of the mycobacteria throughout the oil mixture. The antigen solution was then added as described above. The various procedures for preparation of adjuvant mixtures were adapted from Herbert (1973).

2. IMMUNISATION PROCEDURES

Birds

Intravenous inoculations were made into the bra chial vein of the wing. The bird was first immobilised by pinning strips of cloth over it to a cork board, so that the head and body were lightly but firmly held (Eslami, 1973) and the wing vein exposed. The area of skin over the vein was swabbed with 70% alcohol and the injection made with a suitably sized needle (usually size 25G). Intramuscular injections were given into the left or right breast (pectoral) muscle approximately 1cm on either side, horizontally, from the 'V' of the breast-bone.

Rabbits and Sheep

To raise antisera in rabbits and sheep, injections were made intramuscularly into both left and right calf muscles in order to utilise the maximum number of draining lymph nodes. Assistance was required in holding the animals during the injection. Rabbits were wrapped firmly in a towel with the left or right leg

/ free. Sheep were backed into a corner and pulled back on their haunches by an assistant so that the legs were uppermost. Primary injections of antigen (usually 1mg) were given in Freund's complete adjuvant containing 1 - 2 mg of mycobacteria. A second booster injection of antigen (1mg) in saline was given 4 weeks later and the animals bled at 7 and 10 days after the second injection. Further booster injections were given at two month intervals and bleedings taken as above.

3. COLLECTION OF BLOOD SAMPLES, SEPARATION AND PRESERVATION OF SERA

Birds

The animals were immobilised as for immunisation (above) and blood samples collected from the bra chial vein through a 25 gauge needle inserted in the opposite direction to the flow of blood. 1 - 2ml of blood was collected from young chickens and proportionately more from older birds. A cotton bud was held over the vein to prevent a haematoma.

The blood samples were allowed to clot and contract in a 37^{o}C water-bath for 1 - 4 hours and ringed with a flame-sterilised metal spatula, to ensure complete contraction. The clotted blood samples were then centrifuged at 750g for 10 - 15 minutes, sera removed with a pasteur pipette and stored at -20^{o}C in sealed containers.

Rabbits

Rabbits were bled from the marginal ear vein. The animals were tightly wrapped in a towel with only their heads protruding. The hair over the vein was shaved and the ear lightly swabbed with xylene to cause vasodilation. The vein was compressed at the base of the ear, an incision made proximally across the vein and the

/ blood allowed to drip into clear, sterile containers. Approximately 50mls of blood could be collected at a time. After collection, a small cotton swab was pressed over the cut and held for a few minutes to stop the flow of blood. The ear was then washed with soap and water to remove xylene.

Clotting and contraction procedures were carried out as detailed above. The sera were stored at -20°C.

Sheep

Sheep were bled from the jugular vein. An assistant was required to sit on the sheep and to stretch the neck by holding the head upright. The wool over the vein was clipped and a needle attached to plastic tubing (Travenol Laboratories, Norfolk, England) inserted into the vein. Pressure was applied to the vein just below the needle. The blood was collected via the plastic tubing into clean, sterile universal containers. About 500 - 750ml could be collected at one time.

Clotting and contraction of the collected blood was carried out as before, the sera dispersed in 20ml aliquots and stored at -20°C.

4. RADIO-IODINATION OF PROTEINS

Proteins were radio-iodinated by the direct oxidation technique of Hunter and Greenwood (1962) using chloramine T and thio-sulphate-free iodine-^{131} or iodine-^{125} (Radiochemical Centre, Amersham, England). In general 1mg protein in 0.1ml PBS was added along with 0.25mg chloramine T in 0.25ml PBS to 1mCi iodine-^{131} or iodine-^{125}. The mixture was manually shaken and allowed to interact for 3 minutes before adding 0.12mg sodium metabisulphite in 0.05ml PBS and 2mg potassium iodide in

0.2ml PBS. Free iodide ions were separated from the labelled protein by passage through a G25 (coarse) Sephadex column (30cm x 1cm). The concentration of the resultant iodine-labelled protein was calculated from the optical density at 280nm wavelength in a Unicam SP800 spectro photometer, and the percentage of protein bound radio-label determined by precipitating the protein in 10% trichloroacetic acid and counting the radioactivity of the supernatant and precipitate. In all cases 95% of the radio-iodide was protein bound. The specific activity of the protein was always between 0.1μCi and 1.0μCi per ug.

5. RADIO-ISOTOPE LABELLING OF BURSA AND THYMUS CELL SUSPENSIONS IN VITRO

Autologous cells were used after partial bursectomy, which involved several lobes of the bursa, or partial thymectomy, which involved 2 - 3 thymic lobes. The organs were first immersed in Eagle's Medium (M.E.M.) with admixture of 10% normal adult chicken serum. The bursal or thymic lobes were transected into 5 - 10 slices with fine scissors and the sections teased apart with fine forceps to liberate individual cells.

The cell suspension was incubated in a shaking water bath at 37°C for 90 minutes with 50μCi ^{3}H-adenosine (10μCi/ml, specific activity 4.65 C/mM, Radiochemical Centre, Amersham, England) in a volume of 5mls. After incubation, the cells were washed three times in Eagle's Medium, counted in a Neubauer counting chamber, and adjusted to a final suspension of 5 x 10^{7} cells in 1ml PBS, prior to reinjection.

Individual birds were treated in the above manner, each receiving cells from its own bursa or thymus. Injections of the cells were made into the wing vein in a volume of 1ml.

6. SURGICAL PROCEDURES

Partial Bursectomy

With the bird under open ether anaesthesia, the bursa was exposed through an incision 2cms long midway between the dorsal border of the cloacal vent and the top of the coccyx. Several lobes of the bursa were excised and placed in a petri-dish containing Eagles Medium (M.E.M.) with a mixture of 10% normal chicken serum. The skin incision was closed by a continuous silk suture.

Partial Thymectomy

With the bird under open ether anaesthesia the thymic lobes were exposed through a dorsal mid-line incision along the whole of the neck. Two to three thymic lobes were revealed by blunt dissection and gently removed. The skin incision was closed by a continuous silk suture.

7. MEASUREMENT OF SERUM 7S - GLOBULIN LEVELS IN CHICKENS

Serum 7S-globulin levels were measured by the radial diffusion method of Mancini, Carbonara and Heremans (1965). Sheep anti chicken-7S-globulin was mixed with molten agarose (Behringwerke, Marburg, Germany) at 60^{o}C and then poured into a warmed petri dish on a strictly level bench. When set a series of wells were cut in the agar with a sawn-off size 14 bone marrow needle attached to a suction apparatus. 3 wells were each filled with 5µl of 3 different known concentrations of a purified preparation of chicken 7S-globulin. The other wells were each filled with 5µl of test anti serum. After 48 hours incubation at room temperature in a damp chamber (to prevent the agar drying out) an opaque circle of precipitate was found to have formed round each well.

The diameter of this was proportional to the concentration of 7S-globulin in the well. The amounts of 7S-globulin actually present in the test antisera were calculated by comparison with the result obtained for the three standard solutions of 7S-globulin.

8. MEASUREMENT OF SERUM ANTIBODY LEVELS IN CHICKENS IMMUNISED AGAINST HSA

Serum antibody levels were measured by means of the Farr test (Farr, 1958). This test relies on the differing solubilities of HSA and antibody in saturated ammonium sulphate. The results are expressed as the antigen-binding capacity of the antiserum. HSA was trace-labelled with iodine-^{131} (obtained as preparation IBS-30 from the Radiochemical Centre, Amersham, England) by the direct oxidation technique of Hunter and Greenwood (1962), and used at a concentration of 0.6µg per ml.

Tubes containing undiluted test serum and test serum diluted from 1 in 2, to 1 in 128 in 10% normal chicken serum, in 0.1ml volumes, were set up. Subsequently 0.2ml of the HSA-I^{131} solution was added to each tube and the tubes stored overnight at 4^{0}C to allow the antibody to combine with the antigen. 0.2ml of saturated ammonium sulphate was then added to each tube to precipitate out the antigen-antibody complexes from the trace antigen. After shaking for 2 hours at 4^{0}C the precipitates were obtained by centrifuging (4^{0}C) at 850g for 30 minutes and washed once in 0.5ml 40% saturated ammonium sulphate. The final precipitate was dissolved in 0.5ml 0.15M saline. Four tubes containing 0.1ml 10% normal chicken serum were included in the test. One pair was treated as test sera to determine the minimal precipitations of HSA-I^{131} in the absence of antibody. To the other pair of tubes 0.6mls 10%

trichloroacetic acid (TCA) was added to determine the maximum precipitable radioactivity of HSA-I^{131}. The resulting precipitate was centrifuged and washed in 0.5ml 10% TCA and resuspended in 0.5ml 0.15M saline. The washed precipitates from all tubes and the supernates from the normal chicken serum and TCA tubes were counted in a Nuclear Enterprises automatic well-type gamma counter.

Data Processing

The Farr test data were processed by a method developed by Dr. McKay (personal communication) in this department. The percentage of antigen bound is graphed on a linear scale against the dilution factor of antiserum on a logarithmic scale. For every experimental point on the binding curve an estimate $\underline{a}$ of the antigen binding capacity was obtained by means of the formula:

$$\underline{a} = \frac{0.3\ C\ D \log F}{\log\ (1 - 0.3)}$$

where C = final concentration of antigen in reaction mixture

D = final dilution factor of serum in the reaction mixture ($1<D<256$)

F = proportion of antigen remaining free after equilibration ($0<F<1$)

From all eight or so values of $\underline{a}$, a weighted mean $\bar{a}$ was calculated and taken as the best final estimate of antigen binding capacity ABC-30.

$$\text{i.e ABC-30} = \bar{a} = \frac{wa}{w}$$

/ Where W is the weighting factor for each experimental point.

The weighting factors used were calculated by the formula:

$$W = \frac{1}{\left(\frac{100}{F^2} - \frac{179}{F} + 89\right)}$$

(McKay, personal communication)

Weighting is indispensable here since the experimental points near to 100% binding are much less reliable than those nearer the middle of the graph. The formula (on preceding page) (McKay, personal communication) for the weighting factor is based on a statistical analysis of the errors in radio-activity measurements, i.e. two TCA control tubes with gamma counts of about 10,000, and two negative control tubes with counts around 500.

The calculations were performed in one step from raw data using a programmable desk-top calculator. The values of antigen-binding capacity obtained by this method were found to be less sensitive to experimental errors than the usual method.

As a precaution against indiscriminate use of extrapolation, or unconscious use of data containing large experimental errors, the calculator was programmed to print out not only the weighted mean of the antigen binding capacity estimates for a serum, but also the standard error of the weighted mean, estimated from the extent to which the experimental points on the binding graph deviated from a smooth curve.

$$\text{standard error} = \sqrt{\frac{a^{-2}\Sigma w^2 - 2\bar{a}\Sigma aw^2 + \Sigma a^2 w^2}{(\Sigma w)^2 - \Sigma w^2}}$$

Data giving a very large value of standard error were usually discarded.

9. PREPARATION OF FLUORESCEIN ISOTHIOCYANATE-LABELLED IMMUNOGLOBULIN FROM HYPER-IMMUNE ANTISERA

A) Globulin Preparation

The globulin component of hyper-immune rabbit and sheep antisera was precipitated by the dropwise addition of saturated ammonium sulphate to a final concentration of 50%. The mixture was mechanically stirred for 1 hour at 4°C, and then centrifuged at 6000 r.p.m. (750g) for 10 minutes at 4°C. The precipitated globulin was re-suspended in 50% saturated ammonium sulphate, stirred for 15 minutes and centrifuged as before. Next the precipitate was dissolved in 0.15M saline and dialysed against saline to remove ammonium and sulphate ions. The dialysate was changed at least every 8 - 16 hours. Dialysis was complete when sulphate ions could no longer be demonstrated in the dialysate as indicated by a negative precipitation test, i.e.

2ml dialysate + 4 drops 0.1M HCl + 4 drops 2% barium sulphate

$\longrightarrow$ white precipitate due to the formation of barium sulphate

The globulin solution was removed from the dialysis tubing (Visking tubing: Scientific Instruments Centre Ltd., London, England) and centrifuged at 500g for 10 minutes to remove insoluble

/ aggregates. The protein content of the solution was measured on an SP800 Unicam U.V. spectro-photometer at 280nm. An optical density of 1 was assumed to be equivalent to a concentration of 1mg globulin per ml.

B) Conjugation with FITC

Two methods were normally used for the conjugation of the globulin fraction with fluorescein isothiocyanate (FITC) (British Drug Houses Ltd., Poole, England). These were : i) the direct method and ii) the dialysis method.

i) Direct Method

The first method involves the direct addition of FITC to the globulin solution and is based on the technique of Cherry, Goldman and Carshi (1960). FITC (1.5mg) was dissolved in 1.5ml 0.5M carbonate-bicarbonate buffer (pH9.0 - 9.2) and added dropwise to 100mg of the globulin solution, in an ice bath on a magnetic stirrer. After addition, the pH was checked and adjusted to pH 9.0 by adding more buffer, if necessary. The mixture was then stirred for 18 hours at 4°C.

ii) Dialysis Method

The second method followed the dialysis method of Clark and Shepard (1963). The pH of the globulin solution (100mg in 8.5ml 0.15M saline) was adjusted to pH 9.0 by the addition of 1.5ml 0.5M carbonate bicarbonate buffer and dialysed for 72 hours against a solution of FITC 0.1mg/ml in 0.05M carbonate bicarbonate buffer in 0.15M saline.

C) REMOVAL OF UNREACTED FLUORESCEIN

After the conjugation process the resulting mixture contained FITC conjugated globulin and unreacted fluorescein at a high pH. The mixture was passed through a column of 6g G25 Sephadex equilibrated with PBS, to obtain the pure FITC conjugated globulin (pH 7.2).

D) REMOVAL OF NON-SPECIFIC FLUORESCENCE

The resultant conjugate consisted solely of protein linked to fluorochrome. Unfortunately some of the molecules will be over-labelled with fluorescein and have a high negative charge. These tend to be absorbed in a non-specific manner onto tissue and obscure true specific staining. Those over-labelled molecules were removed by passage through a column of DEAE cellulose (1gm per ml conjugate) equilibrated with PBS, and the first peak collected. When necessary the conjugates were concentrated by dialysis against carbo wax followed by dialysis against PBS, after adjusting the sac to the new volume.

E) FLUORESCEIN TO PROTEIN MOLAR RATIO

The optical densities of the fluorescein and protein were measured on the U.V, spectrophotometer at 495 and 280nm respectively, and the molecular ratio of fluorescein to protein calculated from the following formula:

$$\frac{\text{Fluorescein}}{\text{Protein}} \text{ molar ratio} = \frac{4.8}{\frac{OD_{280}}{OD_{495}} - 0.36}$$

Where 4.8 is a proportionality factor obtained from calibration graphs prepared by measuring the OD of solutions containing weighed amounts of mammalian globulin and fluorescein isothiocyanate, and 0.36 is the $\frac{OD_{280}}{OD_{495}}$ ratio for FITC solutions alone.

An FITC : Protein ratio of 1 - 2 was considered to indicate a good conjugate.

The conjugates were stored in test-tubes at $4^{o}C$ and one drop of 12% sodium azide was added to prevent fungal or bacterial growth.

10. MEASUREMENT OF SPECIFICITY OF FLUORESCE NT-ANTIBODY PREPARATIONS USING IMMUNOELECTROPHORESIS AND ULTRAVIOLET ILLUMINATION

To test the specificity of fluorescein-labelled antibody preparations prior to their use on tissues a simple method employing immunoelectrophoresis and ultraviolet illumination was used (Davis et al, 1974). Immunoelectrophoresis was carried out by the method of Scheidegger using conjugated and unconjugated globulin and a variety of antigens. After precipitin lines had developed the immunoelectrophoresis plates were washed in saline for 24 hours to remove unreacted protein. The plates were then viewed using a visible light source and an ultraviolet source. All precipitin reactions were visible under white light while only the fluorescein conjugated globulin precipitin lines were visible under ultraviolet illumination. This procedure served as a useful preliminary check on both the quantity and quality of the fluorescent conjugate before use on tissues.

11. AUTOPSY PROCEDURES

Animals, after being weighed, were killed with an overdose of intravenous sodium pentabarbitone("Nembutal", Abbot Labs., Kent, England). The skin was then cut along the midline from the sternum to the neck and pulled back to reveal the breast. Depending on the experiment the following procedure was taken before tissue samples were removed for histology.

Measurement of Granuloma Weight

If the bird had formed a granuloma at the site of injection of an adjuvant, usually the breast muscle, the entire muscle was carefully dissected away from the sternum and rib-cage and weighed on a 1kg beam balance (W.A. Webb Ltd., Ilford, England). The opposite control breast muscle was also dissected out and weighed and the difference between the two taken as a measure of the weight of the granuloma.

12. HISTOLOGICAL PROCEDURES

Tissue specimens were removed from the dead birds and subjected to the following histological procedures.

A) Routine Histology

The tissue specimens were removed and fixed in 10% formol saline for 1 - 2 days, then dehydrated through alcohol and embedded in paraffin wax. Three to five micron (3 - 5μ) sections of the wax blocks were cut, mounted on slides, rehydrated, and stained with haematoxylin and eosin (H. and E.) or methyl-green pyronin (M.G.P. or Unna-Pappenheim's Stain).

B) FLUORESCENCE HISTOLOGY

Thin sections of tissue were lodged on the side of a thin-walled glass test-tube, which was then sealed with a rubber bung and rapidly frozen by immersion in a freezing mixture of solid carbon dioxide and acetone at -70°C, contained in a Dewar Flask. Three to five micron sections of these frozen blocks were cut in a cryostat at -20°C, picked up on a "warm" (20°C) microscope slide and allowed to air dry for 5 - 10 minutes at room temperature before being fixed in absolute methanol for 10 minutes. After being rapidly dried in a current of air the sections were stained in one of the following ways.

i) To demonstrate the presence of antigen: the single layer or "direct" method

The tissue section was hydrated by dipping the slide in PBS, and the excess solution dried from round the section. Specific FITC - conjugated antibody was applied, as a drop directly over the section and mixed with the over-lying PBS by sucking up and down in a Pasteur pippette. Excess conjugate was returned to the container. The section was left in a moist chamber (a plastic sandwich box) for 1 hour at room temperature. After incubation with the conjugate sections were transferred to coplin jars containing PBS and washed in 5 changes of PBS with gentle mechanical shaking for 5 minutes per wash. The sections were then mounted in 30% glycerol in PBS and the edges of the cover slips sealed with nail-varnish.

ii) To demonstrate the presence of specific antibody: the "sandwich" method

A solution of the antigen (2mg per ml in PBS) was first applied

to the section, incubated for 30 minutes at room temperature and followed after three, five minute washes in PBS by specific FITC-labelled antibody for 1 hour as described overleaf. This "sandwich" technique (Coons, Leduc and Connolly, 1955) will also demonstrate the presence of antigen in the tissue as in the direct method of staining described in part i). However, by comparison of the two adjacent sections, one stained by each method, antibody can be differentiated from antigen by its presence only in the section stained by the two layers of the "sandwich" technique, as well as by the very clear difference between the two types of staining.

iii) To demonstrate the presence of chicken immunoglobulin

FITC-labelled anti-chicken immunoglobulin was applied directly to the tissue section after it had been washed 3 times in PBS for a total of 10 minutes to remove any unbound chicken-immunoglobulin.

iv) Demonstration of acid-fast bacilli in tissue sections using auramine-rhodamine

Heat-killed injected mycobacteria were identified in both cryostat prepared and paraffin wax tissue sections by use of the auramine-rhodamine staining technique. The method used is a slight adaption of that described by Culling (1974). Air-dried, methanol-fixed, cryostat, or rehydrated, paraffin wax sections were stained with filtered auramine-rhodamine at 60^{o}C for 10 minutes, washed in tap water for 2 minutes, differentiated in 0.5% aqueous HCl in 70% alcohol for 2 minutes, washed again and counter-stained with 1 drop of carbol thionin for 2 minutes. They were again washed in tap water for 2 minutes, and mounted in 30% glycerol/PBS and

examined in a U.V. microscope with a BG.12 exciter and OG1 Barrier filter. The bacilli show up as a brilliant gold fluorescence against a dark blue-green background.

C) AUTORADIOGRAPHY

The technique of autoradiography was used to detect the presence of radio-isotopically labelled antigens and lymphoid cells in tissue sections. The basis of this technique is the exposure of a highly sensitive photographic emulsion to radio-active emission. Upon development of the emulsion a latent image of blackened silver grains is obtained, corresponding to the original radio-active sources. This is not a new phenomenon, being first described by Niepce de St. Victor as long ago as 1867 in an account of the blackening produced on emulsions of silver chloride and iodide by uranium nitrate and tartrate.

The basic principle remains the same though recent years have seen development of highly sophisticated emulsions and isotopes. Modern methods of autoradiography and their followers are divided into two main categories, the "strippers" and the "dippers". The first category involves the attachment of previously prepared, thin strips of gelified emulsion to tissue sections on microscope slides. The second involves dipping sections into containers of warmed liquid emulsion to obtain a thin coat all over the slide. The method preferred in this laboratory is the "dipping" technique.

Dewaxed and rehydrated tissue sections, and cryostat sections which had been air-dried and fixed in methanol for 10 minutes, were dipped in Ilford Nuclear Emulsion K5 (Ilford Ltd., Ilford, Essex, England) according to the method of Kopriwa and Leblond (1962) and exposed for varying times. In every case, at least 3 batches of sections from the

same tissue blocks were exposed and each batch developed at different times to determine the optimum exposure time. The slides were processed in Kodak D19B developer (Kodak Ltd., London, England) and fixed in 25% "Amfix" (May and Baker, Cumbernauld, Scotland) in distilled water. The slides were washed in running tap water for 15 minutes before being stained by the Unna-Pappenheim method with methyl-green pyronin (U.P.)

D) COMBINED IMMUNOFLUORESCENCE AND AUTORADIOGRAPHY

i) To demonstrate the presence of two antigens in same tissue section

In order to demonstrate the presence of more than one antigen on the same tissue section the techniques of immunofluorescence and autoradiography were combined. The method employed represents a simplification of previously described techniques, e.g. freeze substitution (Balfour and Humphrey, 1967) and double autoradiography with two different isotopic labels (Van Rooijen, 1972). Cryostat sections which had been fixed for 10 minutes in methanol and stained by direct immunofluorescence for one antigen (e.g. monomeric HGG) were again fixed in 95% ethanol for 15 minutes and allowed to dry in air for at least 30 minutes before being subjected to autoradiography to detect a second radiolabelled protein (e.g. HSA-I-^{125}). The slides were coated with photographic emulsion (Ilford Nuclear Emulsion, K5) at 45^{o}C by the dipping technique of Kopriwa and Leblond (1962). Next the slides were wrapped in black paper and placed in a light-tight container containing calcium chloride as a dessicant and stored at 4^{o}C for 5 days since in this case (experiment no.6.3.) the specific activity of the radio-labelled

protein was high, 10μCi/μg. The slides were processed in Kodak D19B developer and fixed in 25% "Amfix" in distilled water. The slides were then washed in tap water for 15 minutes and transferred to PBS before being mounted in a mixture of PBS and glycerol (30% glycerol). The edges of the coverslips were sealed with nail varnish.

E) TO DEMONSTRATE THE PRESENCE OF RADIO-LABELLED LYMPHOCYTES AND ANTIGEN IN THE SAME TISSUE SECTION

A slight adaptation of the previous technique enabled the observation of I.V. injected in vitro labelled lymphocytes (see page178) and their relationship to I.V. injected antigen. Again cryostat sections are fixed and stained by direct immunofluorescence for an antigen (e.g. HSA) before being processed for autoradiography to detect the radio-labelled (e.g. ^{3}H-adenosine) lymphocytes. The details are the same as in the previous section though longer exposure times are required for tritium (^{3}H) than for HSA-I-^{125}. Exposure times of two weeks to six months were tried. A slight drop in the intensity of the fluorescence is observed with time though at six months the antigen was still clearly visible. It would seem that storage in a dry light-tight container at $4^{o}C$ provides the conditions necessary for preservation of specific fluorescence.

13. MICROSCOPY

A) WHITE LIGHT MICROSCOPY

Observations on routine histological, cryostat or autoradiographic stained tissue sections were made on either a Leitz SM Lux Student microscope with a built-in light source or a Leitz Ortholux microscope

fitted with a tungsten lamp, dark field or bright condensers, and a vertical photo-tube (E. Leitz, Wetzlar, Germany).

B) FLUORESCENCE MICROSCOPY

Again two systems were used. The first was based on a Wild M20 monocular microscope (Wild, Heerbrugge, Switzerland), fitted with dry and oil-immersion objectives and a cardiodal bi-reflecting dark-ground condensor. The ultra violet source for fluorescence microscopy was an Osram HBO 200 high pressure mercury arc (Osram, Munich, Germany) container within a Wild Universal Microscope Lamphouse. A tungsten lamp for white light observations was built into the base of the microscope and could be brought into use by swinging out a mirror transmitting the light from the ultra violet source to the dark field conductor. For fluorescence work the exciter filter was a 2mm BG12 and the barrier filter an OG1 (both Schott and Genossen, Mainz, Germany).

The second system was based on a Leitz Ortholux microscope fitted with an Osram HBO200 high-pressure mercury arc, oil immersion objectives, and a Ploem-type illuminator. White light observations were made with a tungsten lamp, a combination bright field/dark field/phase contrast condenser (402A), and transmitted light path. An advantage of this system was that white light and fluorescence examination could be carried out simultaneously. The exciter filters used for fluorescence microscopy were a 2mm BG12, a 2mm BG38 and a KP490 mirror, and within the Ploem illuminator a beam splitting mirror TK495 with built in suppression filter. The barrier filter was a Schott OG1 (all filters - Schott and Genossen, Mainz, Germany).

14. MICROPHOTOGRAPHY

Two Leica 35mm cameras were used (E. Leitz, Wetzlar, Germany). A Leica M135 could be fitted to the Wild fluorescence system (above) or to the photo tube of the Leitz Ortholux fluorescence apparatus. Black and white photographs were taken on Kodak Tri-X pan (ASA 400, Kodak Ltd., London, England) or Ilford HP4, FP4 or Pan F (Ilford Ltd., Ilford, England). Colour slides were taken on Kodak High Speed E ktachrome (ASA 160). For fluorescence microscopy exposures of 2 - 5 minutes were required. Black and white films were developed and printed in the department and colour films sent to Scottish Colourfoto Ltd., Dumbarton, Scotland for processing.

RESULTS

RESULTS PAGE

1. GROSS PATHOLOGICAL AND HISTOPATHOLOGICAL CHANGES ASSOCIATED WITH ADMINISTRATION OF FREUND'S ADJUVANT EMULSIONS CONTAINING HUMAN SERUM ALBUMIN (HSA) INTO THE PECTORAL MUSCLE OF CHICKENS

1.1 Gross Pathological Changes

The first noticable effect of the Freund's complete adjuvant (FCA) emulsion injected is the development, as early as two weeks after administration, of a firm granular mass at the site of injection. This can be felt by palp⌣ation with the fingers and compared to the opposite, healthy, breast muscle of the bird. This mass or granuloma slowly develops until it may occupy the entire muscle, causing extensive distension of the tissue. In young birds (six weeks or less) this may even cause distortion of the sternum. (See fig. 1.1.a) The development of the granuloma was followed by palp⌣ation and scored on a subjective plus/minus scheme whereby ++++ denotes a considerable granular mass and - no effect to touch (Table 1.1.a).

No such effect was noticed following injection of Freund's incomplete adjuvant (FIA), the muscle remaining firm and elastic to touch and no different in size to the control.

Fig. 1.1.a.Post-mortem appearance of an 18 week old chicken injected 6 weeks previously with FCA, a water-in-oil adjuvant emulsion containing M.avium and Human serum albumin (HSA). Note the swollen appearance of the right pectoral muscle and the distortion of the sternum at the lower end.

Fig. 1.1.b.Deep transection of the affected muscle shown in fig.1.1.a.reveals an extensive, yellow, caseous granuloma affecting the entire length of the muscle. Individual tubercles can be seen running along the centre of the muscle (arrowed).

Table 1.1.a. Gross Pathological Changes Associated with Administration of Freund's Adjuvant Emulsions into the Pectoral Muscle of Chickens.

Bird Number		Day 7	Day 14	Day 22	Day 28	Day 35
	75086	-	-	±	++	++
	87	-	-	++	++	+++
FCA	88	-	-	-	++	++
	89	-	-	++	+++	++++
	90	-	-	++	++	++
	75091	-	-	-	-	-
	92	-	-	-	±	-
FIA	93	-	-	-	-	-
	94	-	-	-	-	-
	95	-	-	-	±	-

(scored on a - to ++++ basis)

Upon autopsy it was possible to obtain a more quantitative estimate of the extent of granuloma formation by dissecting out and weighing both the injected muscle and the opposite (control) breast muscle. The difference in weight between the two reflects the proliferation of tissue associated with the formation of the granuloma (table 1.1.b.) The pectoral muscles were carefully dissected away from the underlying sternum and rib cage and weighed on a top-loading balance before samples were removed for the various histological procedures.

Table 1.1.b.

INCREASE IN SIZE OF FREUND'S GRANULOMATA WITH TIME

	Bird	Granuloma Weight	Control Weight	Time After Injection	Difference In Weight
FIA	73343	22	20	4 Weeks	2.0
"	73344	15	16	4 Weeks	1.0
"	73345	20.5	21	4 Weeks	0.5
"	73346	16	15	4 Weeks	1.0
FCA	73347	28	23	4 Weeks	5
"	73348	40	20	4 Weeks	20
"	73349	25.5	13.5	4 Weeks	12
"	73350	26	15	4 Weeks	11
"	73351	30	17	4 Weeks	13
"	73352	21	9	4 Weeks	12
"	73353	22.5	12.5	4 Weeks	10
"	73354	ND	ND	4 Weeks	ND
"	74019	10	9.0	1 Week	1.0
"	74020	10.5	7.0	2 Weeks	3.5
"	74021	10.5	8.0	2 Weeks	2.5
"	74022	21.5	20.0	4 Weeks	1.5
"	74023	20.0	6.5	3 Weeks	13.5
"	74024	19.0	6.5	3 Weeks	12.5
"	74025	8.5	5.0	1 Week	3.5
"	74026	12.0	7.5	4 Weeks	4.5
FIA	74033	9.5	7.5	2 Weeks	2.0

Graph Showing Increase in Weight of Freund's Adjuvant Granulomata with Time after Administration.

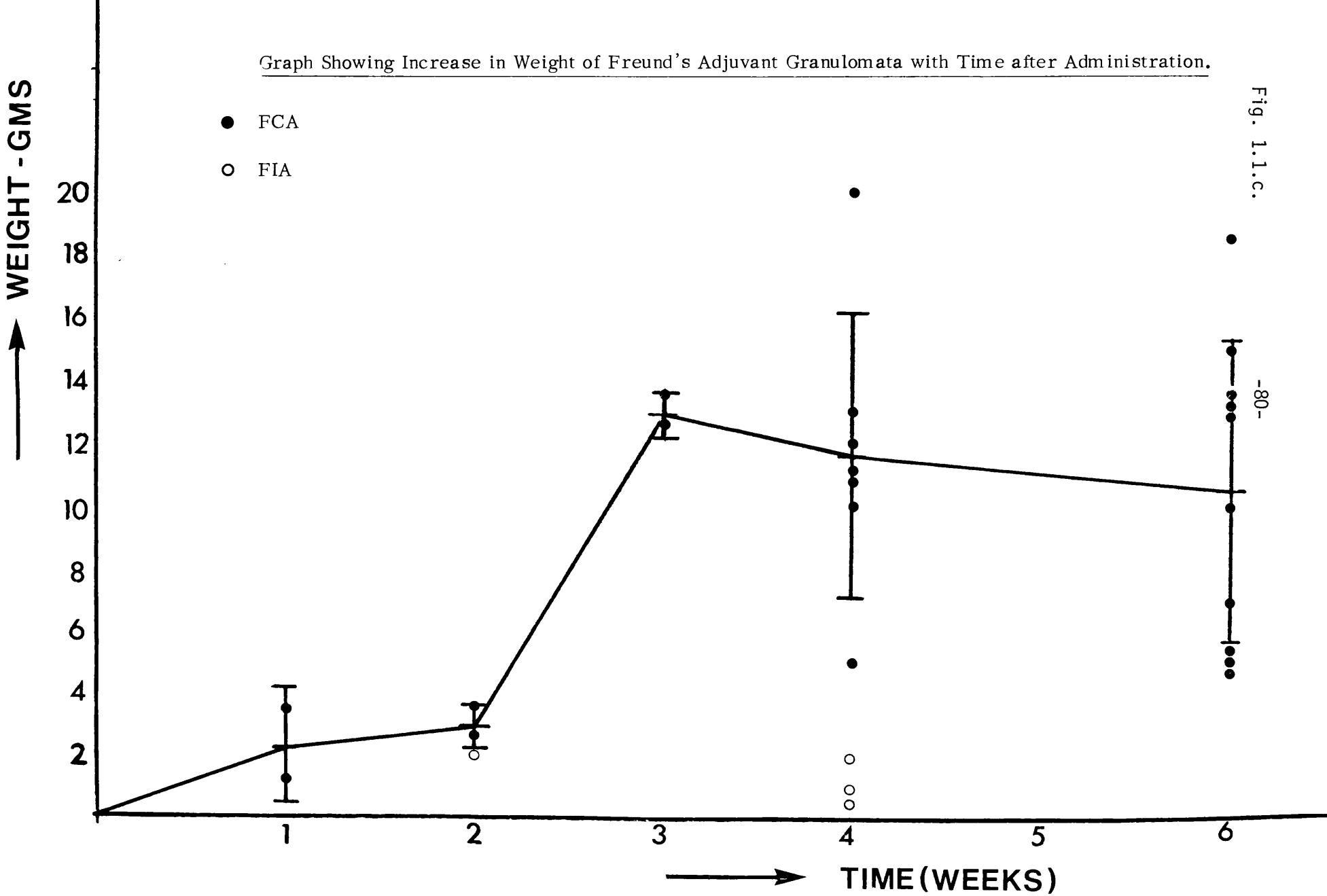


Fig. 1.1.c.

As can be seen from the previous table and from Fig.1.1.c. there seems to be a rapid increase in the granulomatous tissue between the second and third week after injection. This may reflect the sudden development of delayed type hypersensitivity known to occur at this time (White and Herbert, 1976). Thereafter the size of granuloma seems to remain reasonably constant though there is considerable variation in granuloma weight between animals. This variation is probably due to differences in the efficacy of the adjuvant mixtures, some being more stable than others.

FCA: The breast muscles that had received an injection of FCA were discoloured by a greyish-yellow mass underlying the surface and sometimes running the whole length of the muscle, up to 5 inches in length by 2.5 inches wide. When the muscle was transected by a deep incision the granulomatous tissue was revealed as an extensive yellow, caseous , crumbly mass often containing pockets of oily fluid (presumably remnants of the adjuvant mixture) and areas of necrosis. The normal pattern of muscular fibres was totally displaced and destroyed. Occasionally tubercular nodules were found on the surface of the muscle (See Fig.1.1.d) underlying the skin. This may have been caused by a leak-back of the injection mixture along the needle-track. There was a noticable increase in vascularity in the tissues surrounding the affected site. It should be noted that the granuloma did not seem to affect the birds' health to any great extent, their weight was normal, they did not appear to be stressed or try to peck the site of injection.

Despite the outward appearance of normality at one and two weeks tissues taken for examination at these times also revealed a fluid, extensive granuloma. By three weeks after injection the granuloma was

Fig. 1.1.d.

12 week old chicken injected 6 weeks previously with FCA containing HSA. In addition to occupying the pectoral muscle tissue the adjuvant has resulted in the formation of tubercular nodules on the muscle surface (arrowed), possibly as a result of leak-back of injection materials along the needle track. Note the increase in vascularity of this superficial mass.

easily palpable and by four to five weeks the muscles were grossly enlarged and discoloured (fig. 1.1.a.)

FIA: Superficially there was no noticeable effect after an injection of antigen in FIA. (See fig. 1.1.e.) The muscle tissue at all times remained pink, firm and elastic to the touch. An incision however, revealed a very slight oil depot, yellow in colour, running between the fascia of the muscle bands. Close inspection showed numerous small oil droplets apparently encased in fatty membraneous tissue (fig.1.1.f) This was noted as a water-in-oil depot. Early depots of this type tended to be more fluid, and contained free oil but in the later examples the oil droplets had become encased.

1.2. Histopathology:

The appearance of the FIA histological reactions will be discussed first before proceeding to the more complex FCA reactions.

H. & E. and U.P. stained histological sections of incomplete adjuvant granulomas of various ages were examined microscopically. There was little variation with time and the appearances at one week was also typical of later lesions. Running between the fascia of the pectoral muscleswere numerous oil vacuole spaces surrounded by large numbers of 'foamy' macrophages, cells that had ingested large quantities of oil droplets (see fig 1.2.a). Surrounding these oil-macrophage areas were perivascular lymphoid nodules consisting mainly of small lymphocytes, though often included in these nodules were large, perfectly symmetrical, secondary nodules or germinal centres. These were sometimes so numerous that they resembled a string of beads running between the muscle fibres (see fig. 1.2.d.). The germinal centres had the typical splenic

Fig.1.1.e.Post-mortem appearance of an 18 week old chicken injected 6 weeks previously with FIA, a plain water-in-oil adjuvant emulsion containing human serum albumin HSA. There is no superficial evidence of granuloma formation and the injected (right) and control (left) pectoral muscles are similar in appearance.

Fig.1.1.f.Deep transection of the right pectoral muscle shown in Fig.1.1.e. There are no obvious signs of granulomatous formation though close inspection revealed small (1mm), clear, oil droplets lying in the connective tissue between the muscle layers (arrowed).

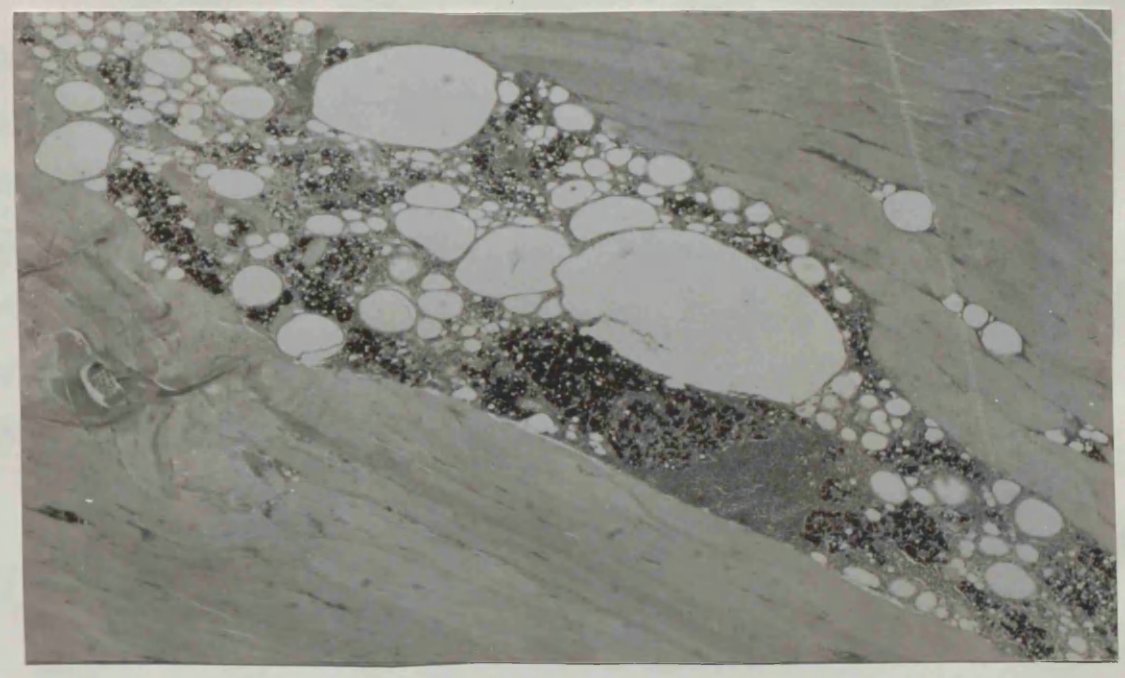


Fig. 1.2.a.

Photomicrograph of section through the breast muscle of a chicken 4 weeks after the local injection of a water-in-oil adjuvant emulsion containing HSA and carbon. The granuloma is confined to the intermuscular septum. Note the numerous white oil spaces.
H & E x 180

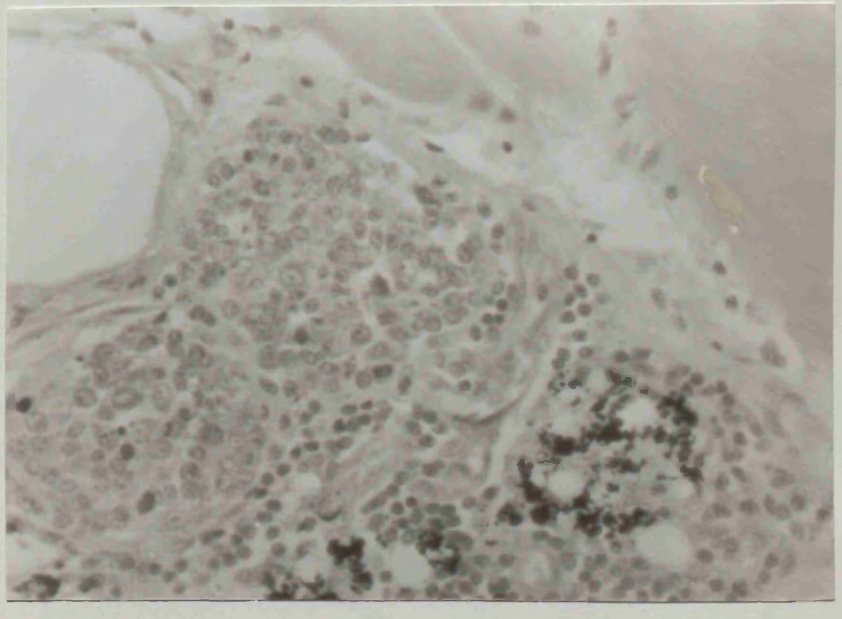


Fig. 1.2.b.

Photomicrograph of small area of granuloma induced by FIA containing antigen (HSA). Note the large oil space at top left bounded by two germinal centres. At the periphery of the germinal centres (bottom right) are numerous smaller oil droplets surrounded by macrophages. The black deposits within the macrophages are carbon particles incorported in the initial adjuvant mixture.
Dominici stain x 720

appearance, distinct circular boundary containing many tightly packed pyroninophilic plasmablasts and small lymphocytes. These centres appeared more numerous at the later times (3 to 4 weeks onwards) but this was difficult to quantitate as there was some difficulty in obtaining a section passing through the entire water-in-oil depot. The lymphoid nodules and germinal centres presumably represent the mural lymphoid nodules (MLN) described by Biggs (1957).

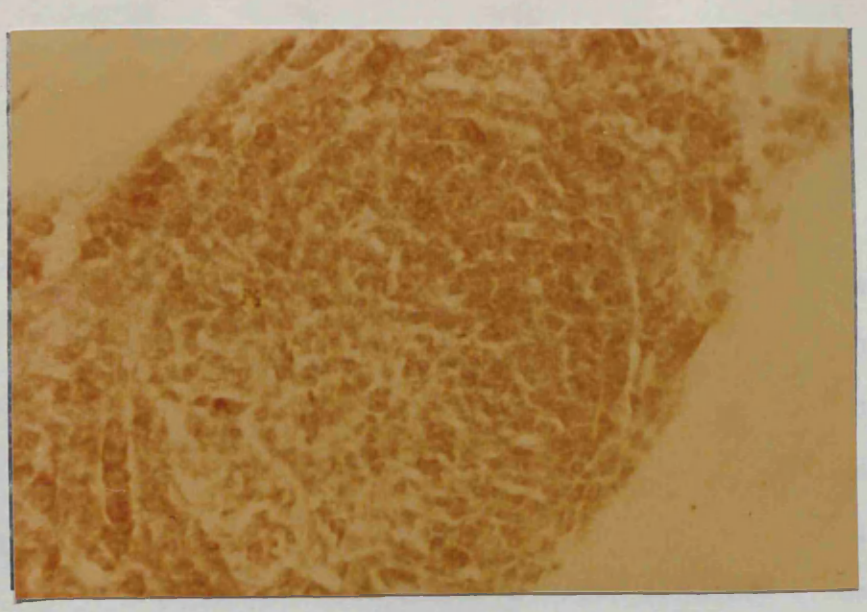


Fig. 1.2.c Photomicrograph of mural lymphoid nodule in lymphatic channel running through pectoral muscle of chicken injected 1 week previously with FIA containing antigen. The unaffected muscle fibres can be seen on either side of the lymphoid mass which includes the distinctive oval mass of a germinal centre.

U.P. X 720

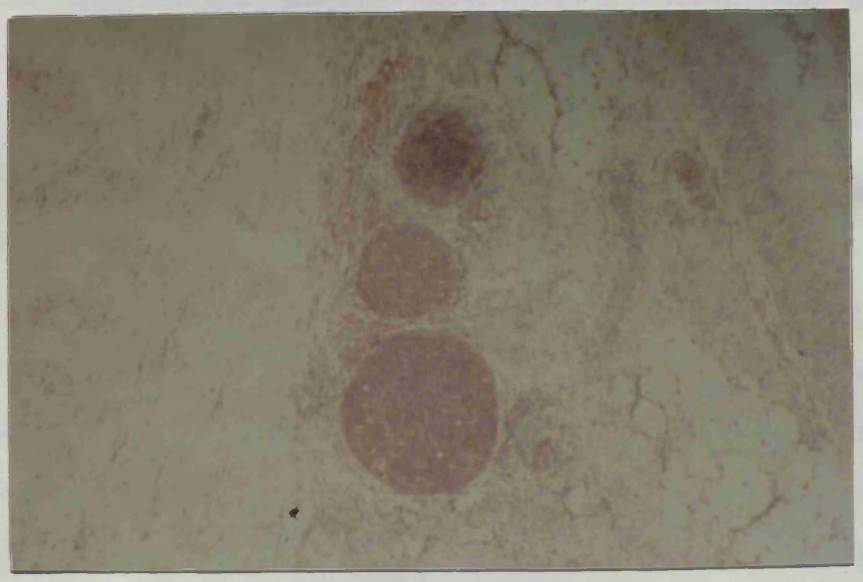


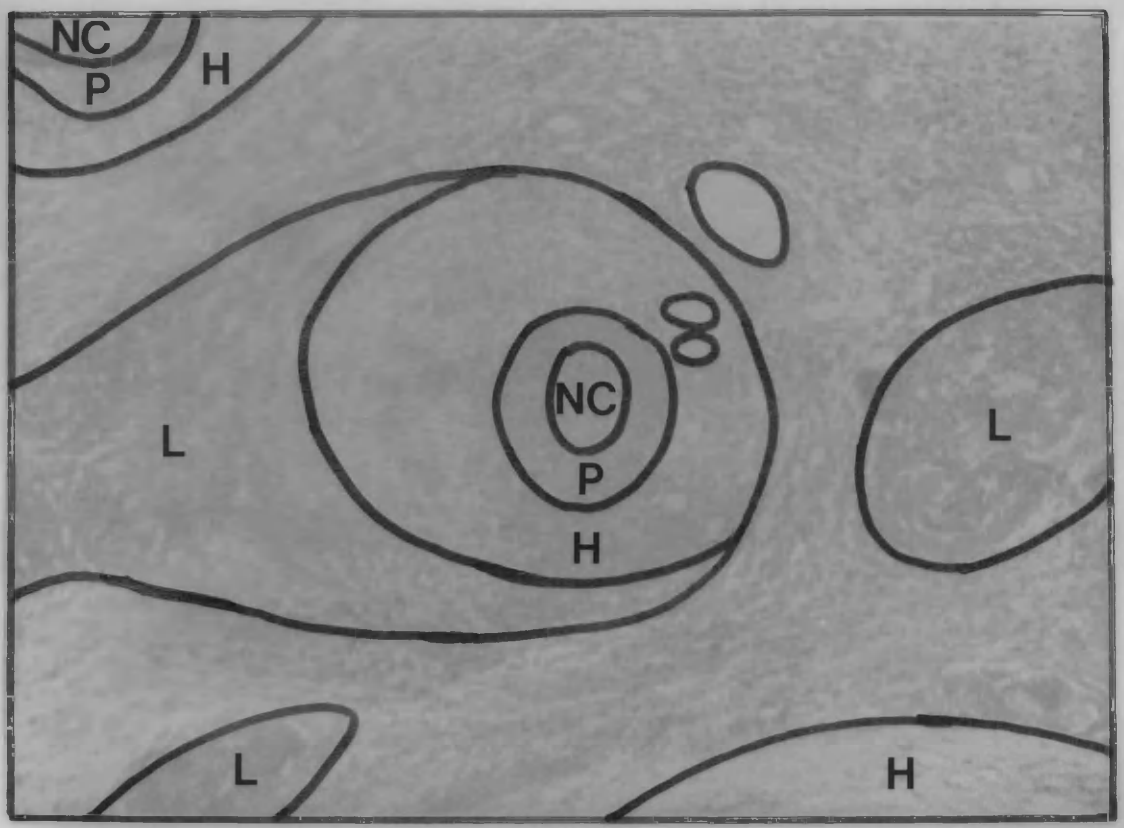
Fig. 1.2.d. Photomicrograph of FIA granuloma 4 weeks after injection. Seen here is a row of three large, compact, well-formed, germinal centres occupying a thin layer of fibrous tissue between muscle bundles.

x 180

FCA:

The histological appearance of the granuloma which forms at the site of an injection of Freund's complete adjuvant changes dramatically with time. At one week after injection microscopic examination of the granuloma site revealed numerous oil spaces scattered throughout muscular tissue surrounded by macrophages, fibroblasts, heterophil leukocytes, plasma cells, and lymphocytes, the latter occasionally forming nodules. The most striking appearance was that of the vast number of small blood vessels infiltrating the early granuloma and encircled in most cases by numerous plasma cells, small lymphocytes and mononuclear cells, suggesting cellular traffic from the blood into the tissue. The small vessels showed a typical high cuboidal endothelium (Fig.1.2.g.) reminiscent of the post-capillary vessels of mammalian lymph nodes. The granulomatous lesions and infiltrates at this early time give the appearance of spreading along defined parallel routes as if the adjuvant mixture is leaking into the lymphatic system.

By the second week the early appearance has changed. The muscle tissue at the site of injection has been grossly destroyed with extensive granulomatous replacement. The granuloma was organised into small, focal lesions of a typical form with an eosinophilic, fibrinoid oval area of central necrosis surrounded by a pallisade of epithelioid and giant cells, heterophil leukocytes and eosinophils, within a wide halo of loose tissue containing large numbers of epithelioid cells and macrophages (Fig.1.2.e.) The focal lesions appear to have derived from the original oil spaces. Bands of small lymphocytes were evident at the periphery of the lesions. Plasma cells were present in large numbers at the edge of the lymphocyte nodules and interspersed with the other cells.



NC - Necrotic centre

P - Palisaded epithelioid cell layer

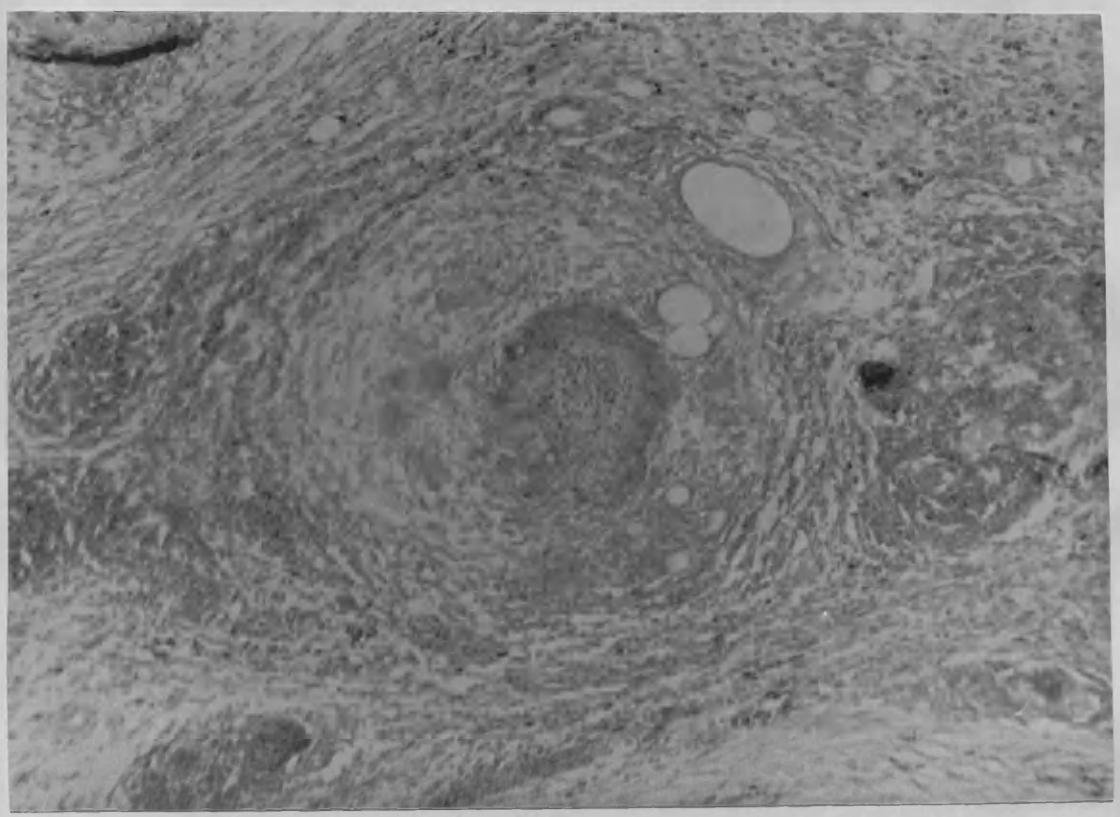
H - Halo of pale loose tissue

L - Lymphoid nodule

Fig. 1.2.e.

Breast muscle of a chicken 28 days after the local injection of Freund's complete adjuvant containing 5 mg M. avium. A central fibrinoid necrotic centre is surrounded by a dense pallisade of epithelioid cells and a halo of pale loose tissue comprised of fibroblasts, epithelioid cells, and lymphocytes. This is ringed by three dense lymphocyte accummulations. The clear white circles are oil droplets.

x 60. U.P.



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x 60. U.P.

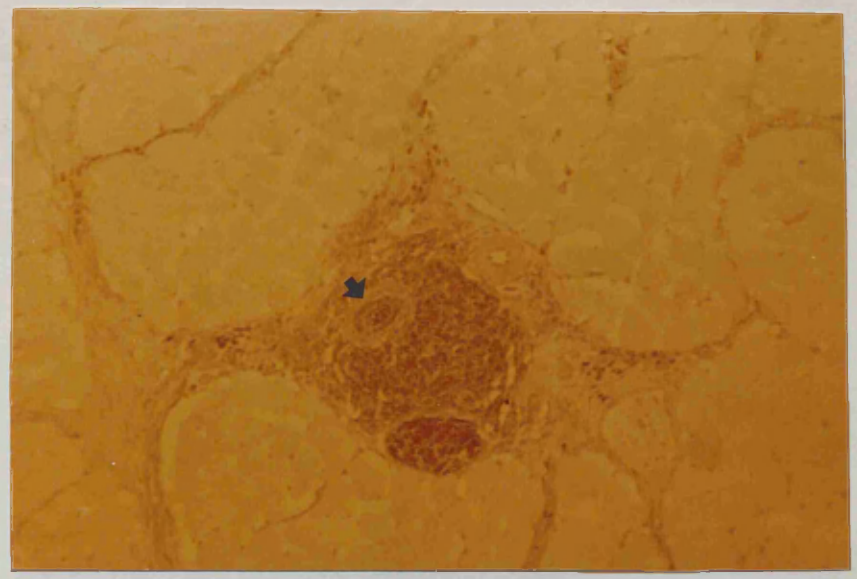


Fig. 1.2.f. Photomicrograph of lymphoid nodule at periphery of granuloma induced by FCA and HSA one week previously. Small blood vessels and lymphatics are seen penetrating the muscle tissue. At the centre of the field is a mural lymphoid nodule with small germinal centre at the bottom and reactive venule at upper left (arrowed).

U.P. X 180

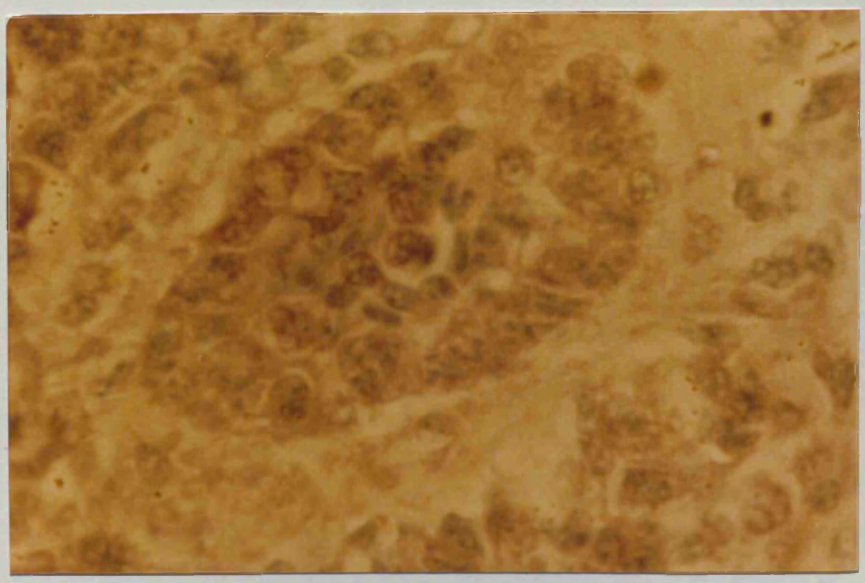


Fig. 1.2.g. Photomicrograph of the reactive venule in fig. f. above. The lumen of the vessel contains several red blood cells and lymphocytes and is bounded by pyroninophilic endothelial cells. The vessel is strikingly similar to the post-capillary vessels seen in mammalian lymph nodes.

U.P. X 1800

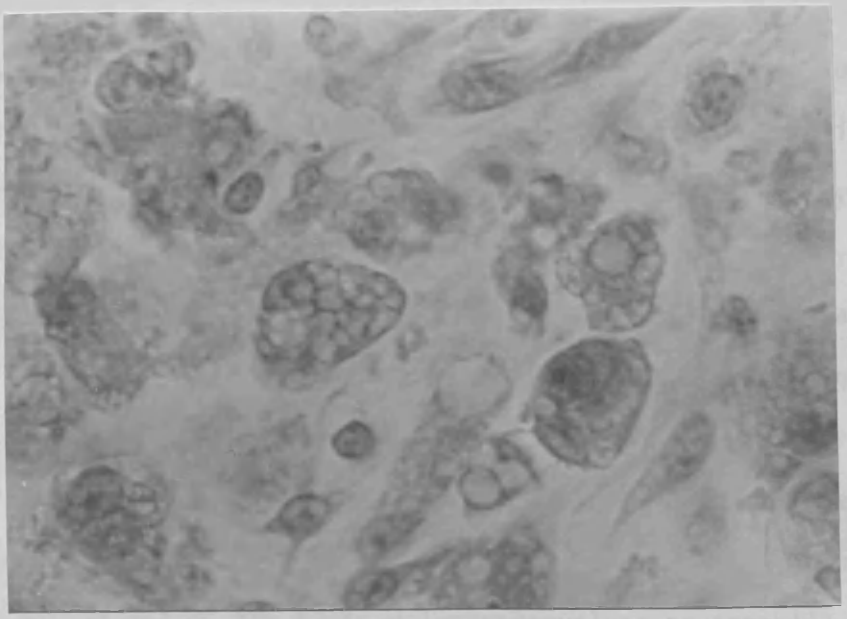


Fig. 1.2.h Section of a granuloma induced by FCA plus antigen one week previously. Large 'foamy' macrophages stuffed with oil droplets can be seen interspersed with fibroblasts and small lymphocytes. H.E. X 1800

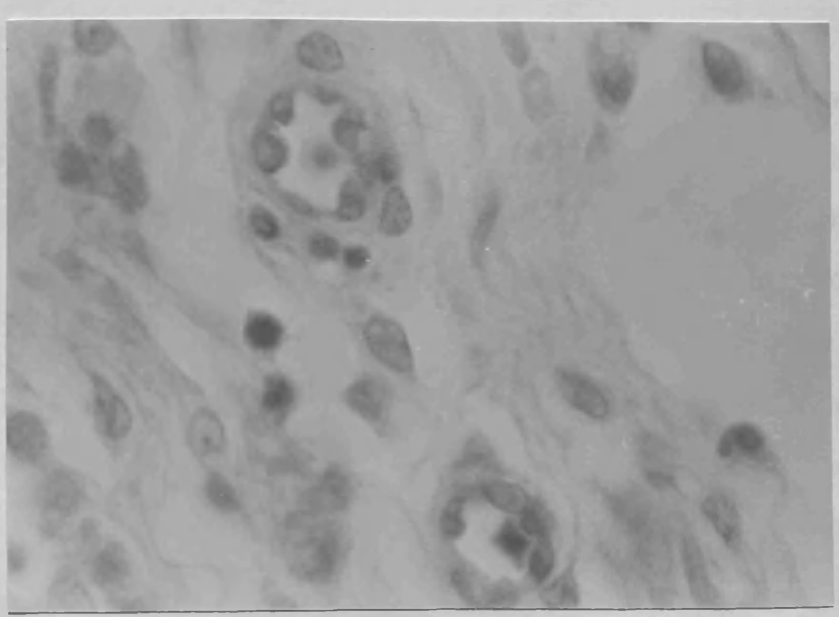


Fig.1.2.i. Section of one week old FCA granuloma showing two small reactive blood vessels with high cuboidal endothelium. To the right is a large oil space derived from the adjuvant injection. H.E. X 720

At the third and fourth week an increase was seen in the number of such lesions and greater organisation of the affected areas so that distinct tissue compartments became evident, i.e. tubercules surrounded by fibroblasts etc., surrounded by lymphoid nodules at the periphery adjacent to unaffected muscle tissue. At these times also there was a sharp increase in the number of plasma cells scattered throughout the granuloma. The necrotic eosinophilic area revealed additional features under the high power lens. At the periphery of the necrotic area and filtering through the ring of epithelioid cells were numerous heterophil leukocytes (the avian equivalent of the human polymorphonuclear neutrophil). These cells have intensely eosinophilic spindle shaped granules in their cytoplasm and are difficult to distinguish from eosinophils. Where these cells had reached the edge of the necrotic area, they could be seen to be in the process of degranulation, scattering their eosinophilic granules inwards to the centre. (fig.1.2.j). This is analogous to the first reports of chemotaxis by Leber (1888) in which polymorphs formed rings around drops of croton oil - obviously there is some highly toxic component at the focal centre of necrosis which causes the polymorphs to degranulate, perhaps a surface active component of the oil. The central areas of necrosis were characterised by a deep eosinic colour identical to the granules of the heterophils. Scattered through this area were numerous cell nuclei, remnants of former degranulated heterophil leukocytes.

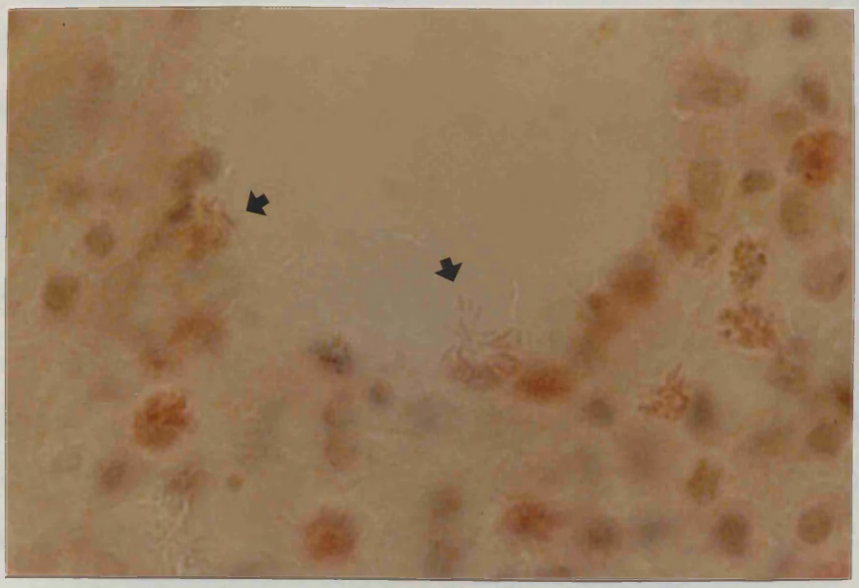


Fig. 1.2.j.

Photomicrograph of FCA granuloma one week after injection of adjuvant emulsion. Seen here is an oil space encircled by epithelioid cells and by eosinophilic heterophil leukocytes. Those heterophils that have reached the oil-cell interface are seen in the act of degranulating, releasing their spindle-shaped inclusion bodies into the centre (arrowed).

H.E. x 1800

By the fourth week the granuloma possesses a full complement of reticulo-endothelial cells, organised into separate functional areas, such that the tissue assumes the appearance in parts of the splenic, or lymph node architecture, with a modified vasculature, lymphoid nodules and plasma cell differentiation. This kind of splenic analogue has also been noted in other species given Freunds complete adjuvant, for example in the sheep (Smith et al. 1970), the guinea pig (Askonas and White, 1956), and the rat (Spector and Lykke, 1966).

There is little change in the histological picture at twenty weeks after injection, suggesting that from the fourth week onwards the granuloma achieves some kind of dynamic equilibrium. The granuloma was slightly reduced in size compared with the earlier examples but the histopathological picture was essentially the same. Large tubercular lesions were evident with necrotic centres, surrounding pallisaded epithelioid and giant cells, and encircled by a variable complement of lymphoid nodules, plasma cells, macrophages and fibroblasts.

Incidence of local germinal centres in Freund's incomplete and complete adjuvant granulomas

One unexpected result of this study, in view of the existing literature, was the observation of local germinal centre formation in granulomas. From the first week after injection onwards very few intact germinal centres were seen in FCA lesions, in direct contrast to the FIA granulomas,

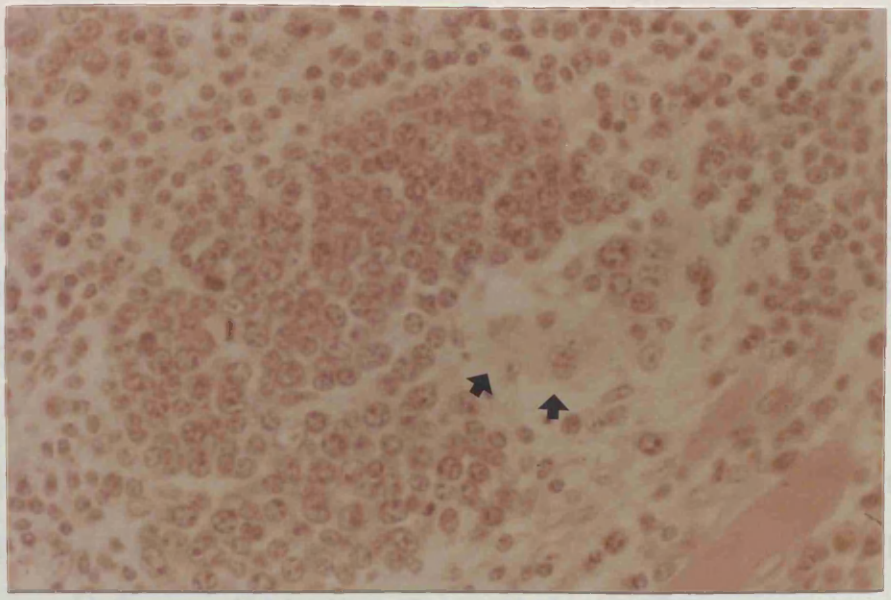


Fig. 1.2.k.Photomicrograph of granuloma at site of FCA emulsion injection. The central aggregate of large plasmablast-like cells resembles a germinal centre. At the right hand side can be seen a small granulomatous reaction. (arrowed) At the lower right hand corner are remains of muscle fibres.

H & E X 720

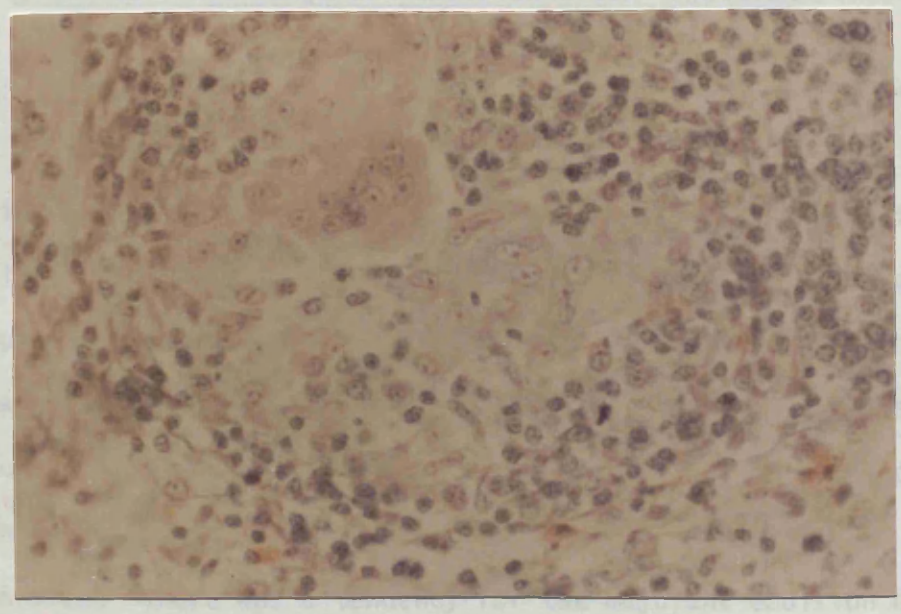


Fig. 1.2.l.FCA granuloma at 5 weeks after injection of emulsion. Lymphoid cell aggregate which has been invaded by numerous epithelioid and giant cells. The distinct boundary at the top edge of the aggregate suggests that this might have been a germinal centre.

Dominici X 720

which were characterised by numbers of large, well-defined centres, developing along the fascial planes between the muscles. Instead bizarre lymphoid aggregates were seen in the FCA reactions, outlined by a distinct boundary similar to those normally seen enclosing germinal centres, but invaded at the periphery by epithelioid or giant cells. (Figs.1.2.k/l). Often these aggregates were identical in appearance to germinal centres except for the epithelioid cell inclusions. It is possible, therefore, that these aggregates represented germinal centres which had been affected by a granulomatous reaction at their periphery with subsequent invasion by epithelioid and giant cells.

1.2.2. Distribution of adjuvant emulsion after injection

At early times (1 week) after inoculation of adjuvant emulsion much of the injection mixture was still microscopically evident in both FIA and FCA animals as clear or white oil droplets at the site of injection and spreading along the fascial planes of the breast muscle (Fig. 1.2.a) Microscopically it was seen at these early times that the oil droplets were surrounded by "foamy" macrophages packed full of oil droplet- containing phagosomes. Macrophages could be distinguished which had phagocytosed oil droplets many times their own size. There was a tendency for the adjuvant emulsion droplets to localise round vessels and spread along lymphoid cell bands running parallel to the long axis of the muscle (Fig1.2.a) This arrangement suggests a gradual drainage and involvement of local lymphatics but

these structures are not easily identified. It is known, however, from the work of Drinker and Yoffey (1941) that lymph capillaries usually exist wherever fascial planes enter the muscle, and that these capillaries empty into trunks which run with the blood vessels. White (1973) has stated that oil adjuvants escape quickly into the lymphatic system in both rabbits and guinea pigs and presumably the same is true of the chicken.

Later granulomata revealed many oil spaces scattered throughout the affected tissue. In FIA animals the oil spaces were still fairly localised and found close together tightly bound by an extensive macrophage-lymphocyte cell infiltrate with occasional lymphoid nodules and germinal centres. In FCA animals the oil spaces are found throughout the entire pectoral muscle and in most cases have become encased within a thick boundary of epithelioid and giant cells. The severity of the FCA reaction probably accounts for the greater spread of adjuvant emulsion in these animals whereas the FIA reaction mixture is more easily confined.

1.2.3. Distribution and degradation of mycobacterial component of FCA in granulomas

The distribution, breakdown, and persistence at the site of injection of the bacterial component of bacterial/oil adjuvants has been performed in great detail by a number of groups (see review by Ginsburg et al. 1975) using highly specific labelling techniques. The following experiments were not designed to achieve such sophistication

but rather to give a general picture of the distribution of the M. avium heat-killed bacilli in the adjuvant granuloma and at other sites in the tissues. Accordingly frozen tissue sections were obtained from complete Freund granulomas of different ages and stained with auramine-rhodamine and examined under the U.V. microscope. Acid-fast bacilli such as M. avium are seen as bright, golden rods under these conditions against a dark background. Cryostat sections were used after methanol fixation as opposed to paraffin wax sections to exclude the possibility that the routine histological dewaxing process would remove most of the adjuvant mixture and therefore the incorporated mycobacteria as well.

At one week the entire cross-section of the granuloma was seen to be occupied by a honeycomb of dense pockets of the golden staining M.avium bacilli (Fig.1.2.m)These pockets ranged in size from a few microns to 1 mm in size and presumably reflect the dispersion of the adjuvant oil droplets in the tissue after injection. In some cases single bacillary rods could be distinguished while in others the rods were too numerous to be counted. Surrounding these pockets and in the process of ingesting the bacilli were sheets of large phagocytic cells. At the periphery of the granulomatous reaction could be seen strands of unaffected muscle tissue (Fig.1.2.m.)

Running through the granuloma and the surrounding muscle fibres were numerous lymphoid bands or channels. Upon detailed examination of these bands bacilli could be seen either as clumps within oil droplets or as individual rods within large phagocytic cells. The

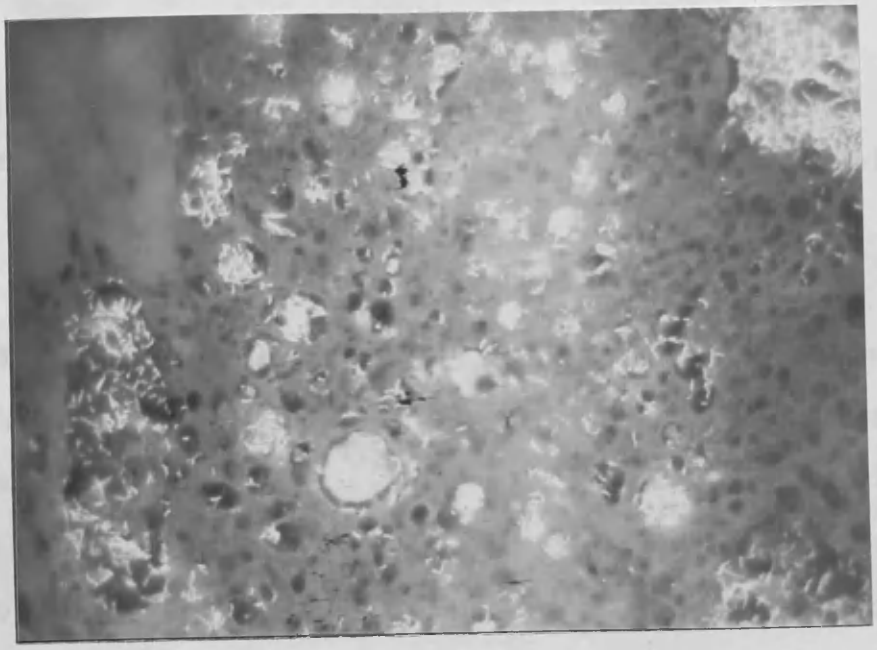


Fig.1.2.m Photomicrograph of FCA granuloma at one week after inoculation of emulsion. The section has been stained by the auramine-rhodamine method for detection of acid-fast bacilli. The entire area of the granuloma is occupied by dense pockets of bright golden bacilli enclosed in oil droplets. At the upper left is unaffected muscle tissue.

Auramine-rhodamine X 720

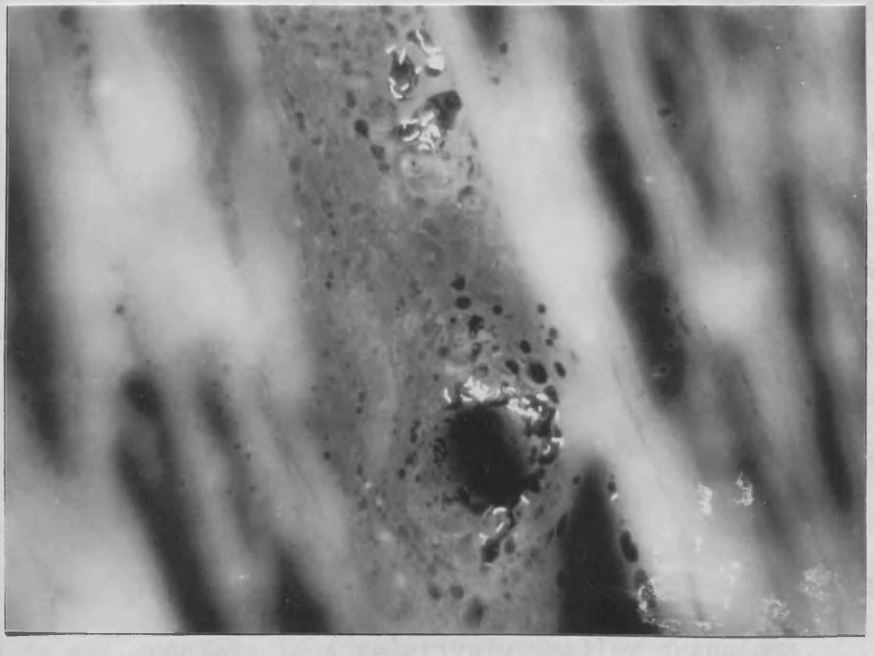


Fig.1.2.nSection through FCA granuloma at one week after inoculation. Lymphatic channel dividing muscle fibres. Situated along the length of the channel are pockets of mycobacteria associated with oil droplets, which have drained from the injection site.

Auramine-rhodamine X 720

clumps of bacilli were staggered along the length of the lymphoid areas as if the oil droplets were draining into the lymphatics (Fig.1.2.n)In blood vessels, by contrast, individual rods could be clearly seen in the cytoplasm of phagocytic cells (Fig.1.2.o)How these bacilli have gained entrance to the blood vascular system is not clear, perhaps as a result of extensive damage to vessel walls.

The distribution of the mycobacterial rods shows little change in the following weeks. The clumps of bacilli become less circumscribed, some coalesce to form large oil spaces which become sealed off by a wall of macrophages and epithelioid cells. The number of individual rods lessens and large phagocytic cells containing golden staining granules became evident at the periphery of the mycobacterial clumps and oil spaces. These cells often gave the impression that they were actively phagocytosing the mycobacteria. Lymphatic vessels were seen at the periphery of the reaction sites and could be seen to contain cells with either whole bacillary rods or round golden granules (Fig.1.2.n)The overall impression gained was of a slow but steady process of degradation of the mycobacteria in progress, and that given time the granuloma would eventually resolve completely.

Several birds were not killed until 20 weeks after FCA inoculation. Though there was little external evidence of a granuloma at the site of injection in those birds, histological examination of the tissues removed at post mortem again showed considerable numbers of mycobacteria persisting in oil droplets in a relatively smaller granuloma.

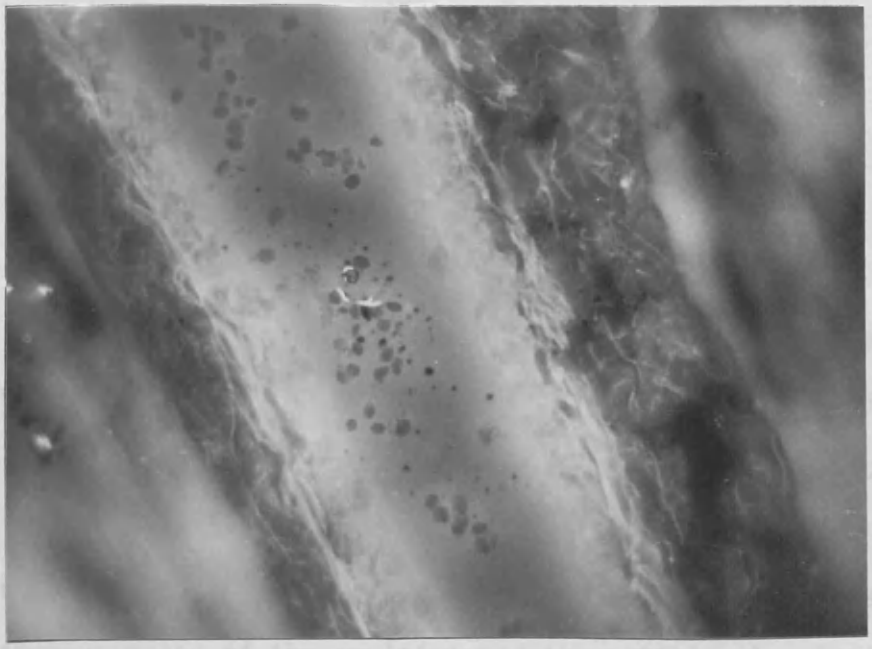


Fig. 1.2.o.

Longitudinal section through a vessel in a one week old FCA lesion. Several mycobacteria (seen as white rods) can be identified in the vessel lumen within phagocytic cells. Also visible are numerous small oil droplets (black).

x 720 auramine-rhodamine

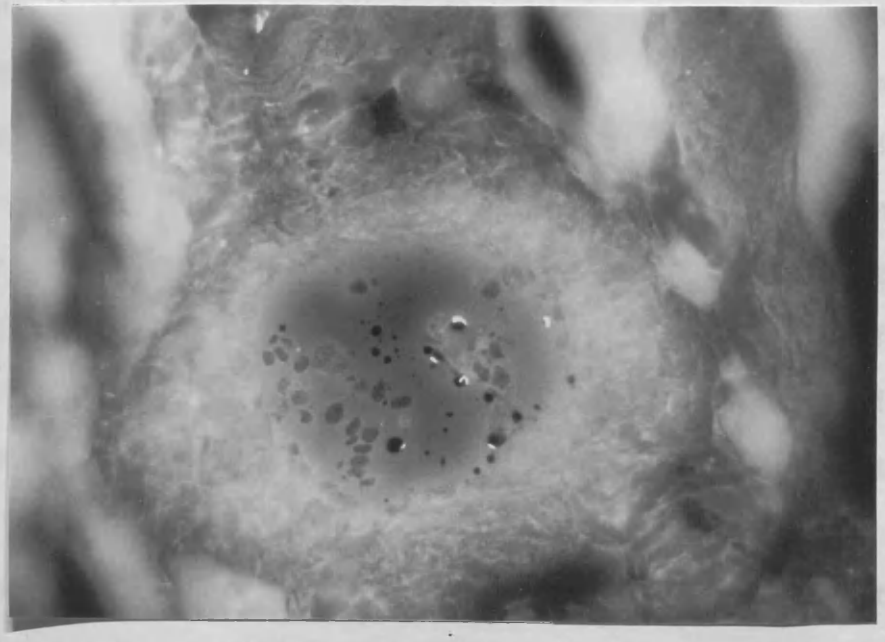


Fig. 1.2.p.

Transverse section through a vessel in a one week old FCA lesion. Six mycobacteria (seen here as white rods) can be seen in oil droplets either free in solution or within phagocytic cells.

x 720

1.2.4. Distribution of an incorporated protein antigen (HSA) in adjuvant mixture

The distribution of the mycobacteria in the granuloma which formed at the site of injection of Freund's complete adjuvant has been discussed in the previous section. It was shown that bacilli were still evident in the granuloma 20 weeks after administration. To assess the adjuvant effect of the FCA a small quantity (40µg) of a protein antigen, human serum albumin (HSA), was also incorporated into the aqueous phase of the emulsion and the antibody levels in the blood monitored over a period of 1 - 7 weeks. In view of the original suggestion by Freund (1956), that a major cause for adjuvant action was the slow release of antigen from a depot at the site of inoculation, providing a constant antigenic stimulus, the distribution of HSA in the granulomas was also followed.

Using the fluorescent antibody technique, cryostat tissue sections were examined from granulomas of 1 to 20 weeks of age, after staining with specific sheep anti-HSA antibody. At one week small quantities of HSA antigen were seen in oil droplets scattered throughout the granulomatous sites of both FIA and FCA injections. The quantities of injected antigen were small (40µg) so the staining pattern was faint. Surrounding the oil droplets macrophages with phagocytic inclusions of HSA were visible. Very little difference was noted between the FIA and FCA reactions with regard to HSA localisation at this time. At the second, third, and fourth week the antigen localisation pattern was similar to the one week appearance though the quantities of HSA were progressively smaller. The central region of the granulomas

still showed brightly staining depots of antigen but the surrounding tissue showed less specific fluorescence. At 20 weeks after injection it was just possible to detect HSA though the amounts were extremely small and confined to an occasional oil depot. It seems likely that the level of sensitivity of the fluorescent antibody techniques prevented a more detailed analysis of remaining antigen.

It was evident however, that in both the complete and incomplete adjuvant granulomas antigen in the form of HSA was still available in depot form as long as five months after administration. The effect of this stimulation on antibody production was next investigated.

1.2.5. Production of specific antibody following adjuvant-antigen administration.

It has long been recognised that adjuvant granulomata may contribute to antibody production. Thus in the rabbit (Askonas and Humphrey, 1958), in the horse (Freund, 1951), and in the chicken (French et al., 1970), there is evidence of considerable production of antibody by the granuloma, in contrast to the guinea pig granuloma which produces very little (Askonas and White, 1956).

French and her colleagues concluded that the major source of specific antibody production in the chicken was the granuloma. This conclusion was based on antibody extraction procedures on various tissues of the chicken, followed by use of the Farr technique to determine the antigen-binding capacity of the extracts and serum samples. Tissues obtained from the present study were subjected to a similar analysis in order to correlate the amount of extractable

antibody with the histological appearance of specific antibody and 7S globulin producing cells, using the fluorescent antibody techniques.

1.2.5. i) Serum samples

Antibody production in response to an antigen administered in Freunds complete and incomplete adjuvant has been well characterised (French et al., 1970). The present experiments merely served to to confirm these findings. Serum samples were obtained during the time course of the experiment from groups of birds injected with either HSA in FCA or HSA in FIA and assessed for their antigen-binding capacity (ABC_{30}) to HSA. Each point represents the mean of four animals. As can be seen from Fig. 1.2.q. both FIA and FCA groups respond to the adjuvant-antigen injection with a sharp rise in antibody levels, peaking between the 7th and 14th day. Thereafter there is a slight plateau in antibody production extending through the 22nd day, followed by a sudden logarithmic increase in the FCA birds while the FIA group peaks only slightly at the 28th day before dropping sharply to a very low level at the 42nd day. At this time the level of the secondary peak of antibody in the serum of FCA animals may be 10 - 100 X that of the primary response. As observed in the primary response between the FIA and FCA emulsions, the major effect of the latter is demonstrated considerably later on in the response. These results are in contrast to those observed in other species, for example in the mouse (Herbert, 1973) in which animals inoculated with FIA plus antigen quickly achieve a high serum antibody level which plateaus for at least one year.

TABLE 1.2.5.i.

ANTIBODY LEVELS ** IN SERUM, SPLEEN, AND GRANULOMA SAMPLES FROM BIRDS INJECTED INTRAMUSCULARLY WITH HSA IN COMPLETE AND INCOMPLETE FREUND'S ADJUVANT.

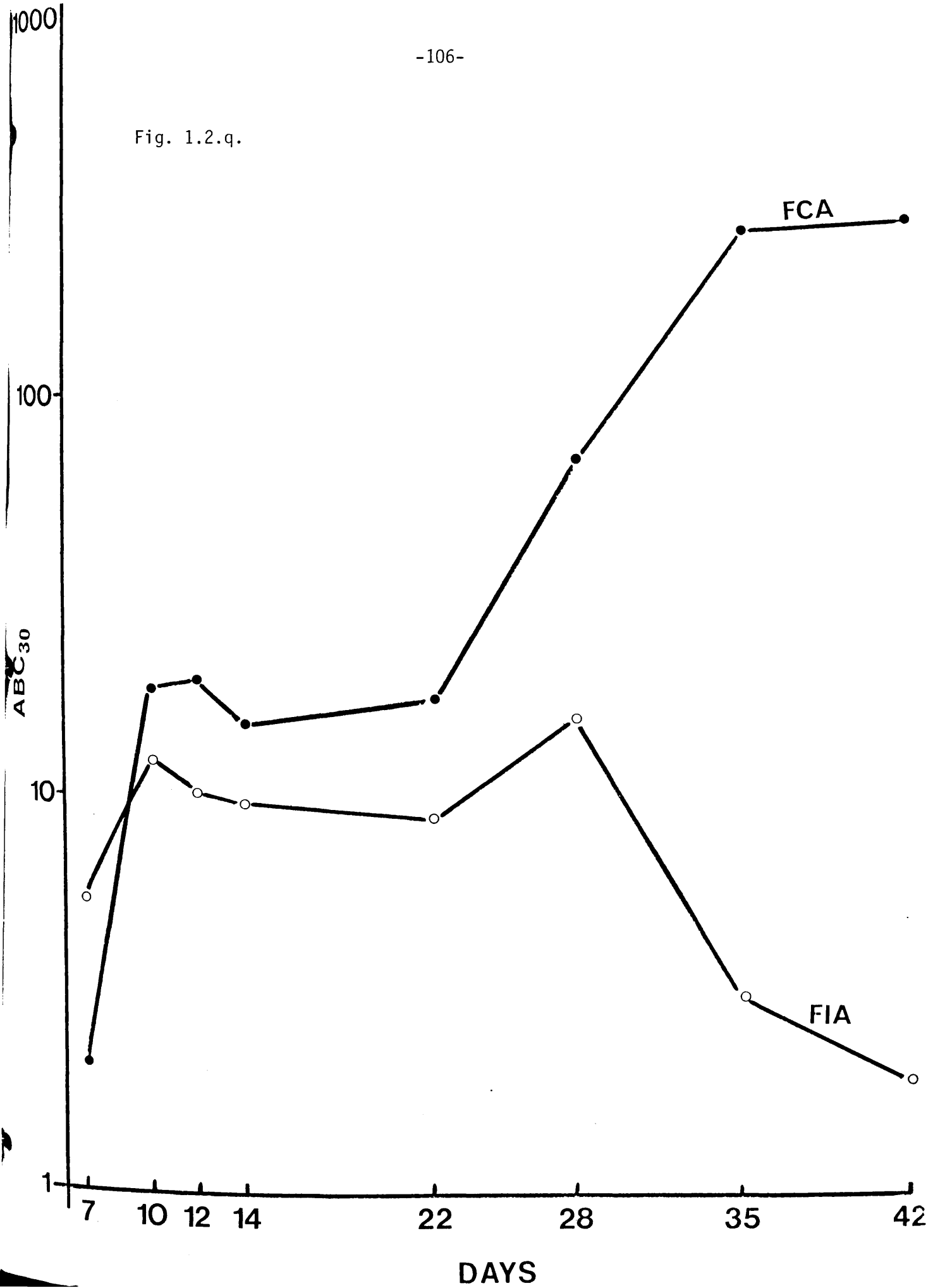
BIRD NO.	TREATMENT	SERUM ANTIBODY LEVEL AT DAY								SPLEEN	GRANULOMA
		7	10	12	14	22	28	35	42	DAY 42	DAY 42
75070	FCA	0.3	6.7	7.1	4.1	6.2	118.1	279.2	220.2	-	282.6
75071	40µg HSA +	1.5	30.6	32.9	27.4	11.7	52.8	-	-	8.8	366.6
75072	2mg M.avium	0.1	4.3	14.1	8.4	7.7	13.5	46.2	269.7	0	138.3
75073	in w/o	6.3	33.4	23.9	19.9	43.5	94.1	-	-	-	312.7
	MEAN	2.05	18.75	19.50	14.98	17.28	69.63	162.7	237.35	4.4	275.05
75082	FIA	0.1	0.5	4.8	5.3	1.5	0.8	0.8	1.0	0	1.7
75083	40µg HSA in	8.8	25.3	17.0	17.3	6.6	6.7	5.3	2.8	0	2.1
75084	w/o	7.6	13.4	14.3	12.6	24.4	49.6	-	-	-	14.3
75085		5.3	10.3	5.0	3.0	2.7	5.8	-	-	-	31.3
	MEAN	5.45	12.38	10.28	9.55	8.80	15.73	3.05	1.9	0	12.35

** = Estimated by the Farr test in µg HSA bound per ml of serum, or per gm of tissue at the 30% level (ABC_{30}).

Fig. 1.2.5.9.

The circulating antibody responses in adult birds injected intramuscularly with 40µg HSA in Freund's complete (●) or incomplete (○) adjuvant. Each point represents the mean value of a group of four animals.

Fig. 1.2.q.



1.2.5. ii) Local antibody production by the granuloma

It was shown by French and her colleagues that the major source of antibody extracted from various tissues of the chicken after FCA administration was the granuloma. In answer to the question of whether this antibody was locally produced or simply a reflection of transport by the blood from other tissues they demonstrated that neither the spleen, bone marrow, liver, lungs, or caecal tonsil could be shown to contain comparable amounts of specific antibody to the granuloma. A second criticism, that the increased amount of specific antibody is a result of binding to antigen at the site of the granuloma, was answered on the grounds that the amount of antigen is so small (40 µg) that one ml. of serum would be more than ample to bind all the HSA.

Groups of birds that had been injected with either FCA or FIA were killed and the granulomas extracted for antibody. The results are shown (Table 1.2.5i) Occasionally a sample of spleen or liver was included as a control. As can be seen from this table very little antibody to HSA could be extracted from the spleen or granuloma of FIA animals. In contrast a very high amount of extractable anti-HSA antibody was found in the granulomas of the FCA group, though not in the spleen or liver.

1.2.5. iii) Effect of adjuvant administration on serum 7S globulin levels

It has been reported (Humphrey, 1963) that use of Freund's adjuvants results in an increase in the total serum globulin levels.

TABLE 1.2.5.ii.

LEVELS OF SERUM 7S GLOBULIN IN CHICKENS INJECTED INTRAMUSCULARLY WITH HSA IN FREUND'S COMPLETE AND INCOMPLETE ADJUVANT.

BIRD NO.	TREATMENT	SERUM 7S GLOBULIN (D^{2}*) ON DAY						
		0	7	10	12	14	22	28
75070	FCA	92.16	96.04	86.49	104.04	100.00	104.04	132.25
75071	"	92.16	95.06	96.04	100.00	116.64	98.01	129.96
75072	"	87.42	82.81	79.21	98.01	102.01	121.00	127.69
75073	"	78.42	102.10	96.04	121.00	82.81	133.40	135.72
	MEAN D^{2}	87.54	94.00	89.44	105.76	100.36	114.11	131.40
	7S mg/ml	12.74	13.68	13.01	15.39	14.60	16.60	19.12
75082	FIA	86.49	86.49	56.25	96.04	96.64	101.12	127.69
75083	"	72.25	89.90	84.60	100.00	96.25	88.36	96.04
75084	"	99.00	100.00	92.16	106.00	88.36	104.04	100.00
75085	"	98.01	104.04	94.09	96.04	90.25	96.14	84.00
	MEAN D^{2}	88.93	94.95	81.77	98.02	91.22	98.89	106.93
	7S mg/ml	12.94	13.82	11.90	14.26	13.27	14.39	15.56

* = square of diameter of precipitin ring in mm^{2}.

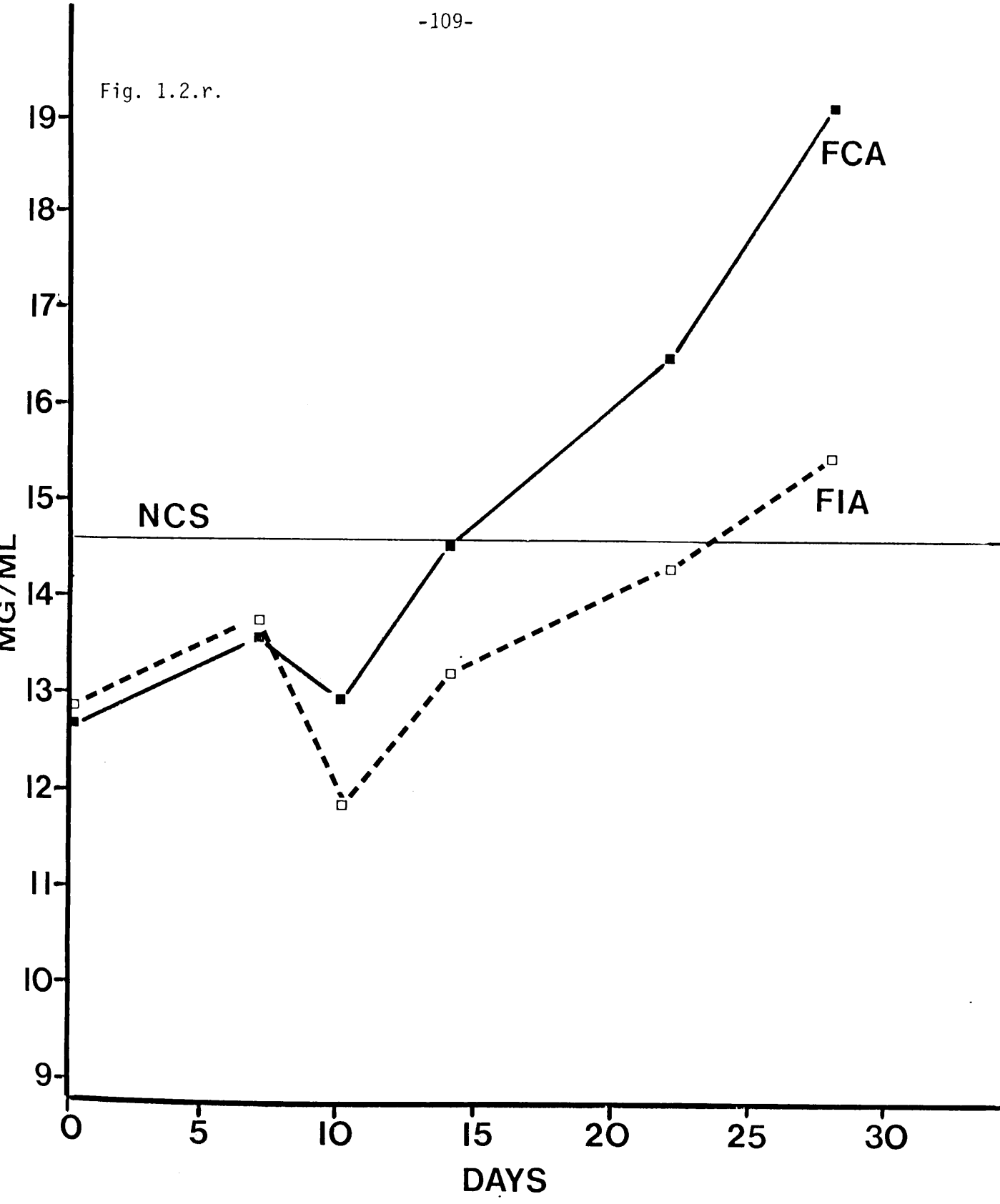
Fig. 1.2.r. Levels of serum 7S globulin in chickens injected intramuscularly with HSA in Freund's complete and incomplete adjuvant.

(■) Freund's complete adjuvant

(□) " incomplete adjuvant

NCS = Normal chicken serum.

Fig. 1.2.r.


19
18
17
16
15
14
13
12
11
10
9
MG/ML
FCA
FIA
NCS
0
5
10
15
20
25
30
DAYS

This was tested in groups of birds given either FIA or FCA by the direct Mancini radial immunodiffusion technique (RID) using sheep anti chick -7S globulin as antiserum. The birds were bled at various times after inoculation and the serum stored at -20°C until used. Fig. 1.2.r.shows the results of the Mancini estimation expressed as mg/ml of 7S globulin against time. There is an increase with time in the total level of 7S in the FCA group as compared with the FIA group.

1.2.5. iv) Histological localisation of chicken 7S-globulin and specific anti-HSA antibody in the spleens and granulomata of birds treated with Freunds complete and incomplete adjuvant emulsions incorporating HSA as antigen

The previous sections have shown that in the granulomata which appear at the site of injection of Freunds adjuvant emulsions there are available large numbers of lymphoid cells, plasma cells, macrophages, a long and persistent availability of antigen and a high level of specific antibody to that antigen. The close relationship between these various elements was next examined by the use of the fluorescent antibody technique in an attempt to clarify the histological sequence of events. Cryostat sections of the spleens and granulomata of both FCA and FIA treated animals were stained with either sheep anti-HSA antibody (using the "sandwich" modification) or sheep anti-chick-7S-globulin (direct method). The stained tissue sections were scanned with a fluorescent microscope to determine the patterns

of localisation of both specific antibody and 7S globulin.

One week after injection of HSA in adjuvant

FCA: Both the spleen and granuloma sections of the FCA treated animals contained numbers of anti-HSA producing plasma cells. In the spleen a moderate number of cells were in the red pulp and grouped round large vessels. The granuloma contained far fewer antibody producing cells and these were either at the periphery of lymphoid nodules or scattered through the sheets of macrophages surrounding oil droplets (Fig.1.2.u). The anti-7S staining revealed greater numbers of immunoglobulin containing cells in the spleen and granuloma as might be predicted from the large number of antigens available to the animals at this time. In addition anti-7S staining showed deposits of globulin in the lumen of vessels in the spleen and on the surface of small lymphocytes in the white pulp areas. A striking pattern was observed with this stain in the splenic and granuloma germinal centres, in which a network of cell surface globulin was seen on the dendritic cells of the centres. A similar pattern was not observed with anti-HSA, either because no HSA was in the germinal centres or because the technique was not able to detect the small quantities of antigen available.

FIA: The staining pattern observed in the FIA treated animal tissues was essentially similar to that noted above. Moderate numbers of anti-HSA plasma cells in the splenic red pulp, a few scattered plasma cells in the granuloma. Anti-7S patterns showed larger numbers of plasma cells in the spleen and in the granuloma.

In addition both splenic and local granuloma germinal centres were observed with a dendritic cell-surface staining pattern of 7S globulin.

Two weeks after the injection of HSA in adjuvant

FCA: No plasma cells making anti-HSA were seen at this time in the spleens of these birds. Again a few scattered plasma cells were observed in the granuloma. In addition a faint pattern of specific fluorescence was noted running between the muscle fibres surrounding the granulomatous site and invading the intercellular spaces of the lesion. The 7S staining demonstrated a few isolated plasma cells in the spleen and larger numbers in the granuloma. The faint anti-HSA fluorescence between the muscle fibres was amplified by the 7S staining so that a network of bright rivers of fluorescent immunoglobulin were seen running through the granuloma and immediate surrounding tissue. 7S but not HSA dendritic cell staining was seen in germinal centres in the spleen but not in the granuloma.

FIA: The spleens of the FIA animals contained very few actively synthesising anti-HSA plasma cells and the granuloma none. Some faint traces of anti-HSA was seen infiltrating the spaces between muscle fibres in the granuloma. The 7S staining pattern was similar though numbers of plasma cells had increased in the spleen and granuloma. There appeared to be an increase in both the size and incidence of splenic and granulomatous germinal centres with 7S dendritic cell surface staining.

Four weeks after injection of HSA in adjuvant

FCA: The appearance at this time of the spleens and granulomata of FCA treated animals was very different. Anti-HSA plasma cells were seen in the spleen, as at the first week, but in this case all the plasma cells were within the confines of germinal centres referred to as "antibody centres". Not all germinal centres contained such cells and not all the cells within the centres were engaged in synthesising antibody but the appearance was nevertheless very striking, with a bright circle of apple-green fluorescent cells in stark contrast to the faint background (Fig 1.2.s). The granuloma section was saturated with antibody such that every channel and space between muscle fibres or granuloma cells was occupied with brightly staining anti-HSA antibody. There were many plasma cells visible scattered throughout the lesion. No germinal centres were seen in the granuloma at this time.

7S staining was an amplification of the above. The "antibody centres" were again seen in the spleen sections with an increase in number and content of plasma cells with antibody. In contrast to the HSA staining pattern, typical 7S dendritic cell surface staining was also seen in other germinal centres in the spleen. The 7S pattern in the granuloma showed an increase in fluorescence over the already saturated HSA pattern, suggesting an extensive localisation of immunoglobulin in the lesion. Almost every space not occupied by muscle fibres or granuloma cells was saturated with globulin (Fig 1.2.v.)

FIA: Again occasional antibody centres were observed in the spleens with both anti-HSA and 7S plasma cells. Outside of these

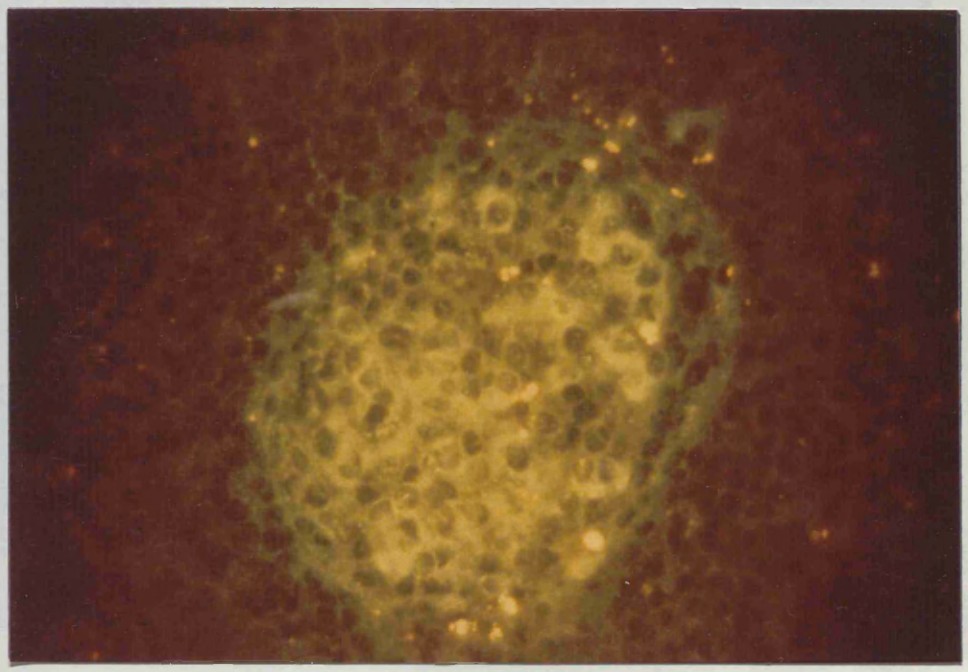


Fig. 1.2.s.

Fluorescence micrograph. Frozen section of chicken spleen 35 days after animal received an intramuscular injection of Freund's complete adjuvant (FCA) containing human serum albumin (HSA) as added antigen. Section stained by the "sandwich" fluorescent antibody method for demonstration of HSA and anti-HSA. The section shows an "antibody centre", a germinal centre in which the majority of cells contain anti-HSA.

x 720

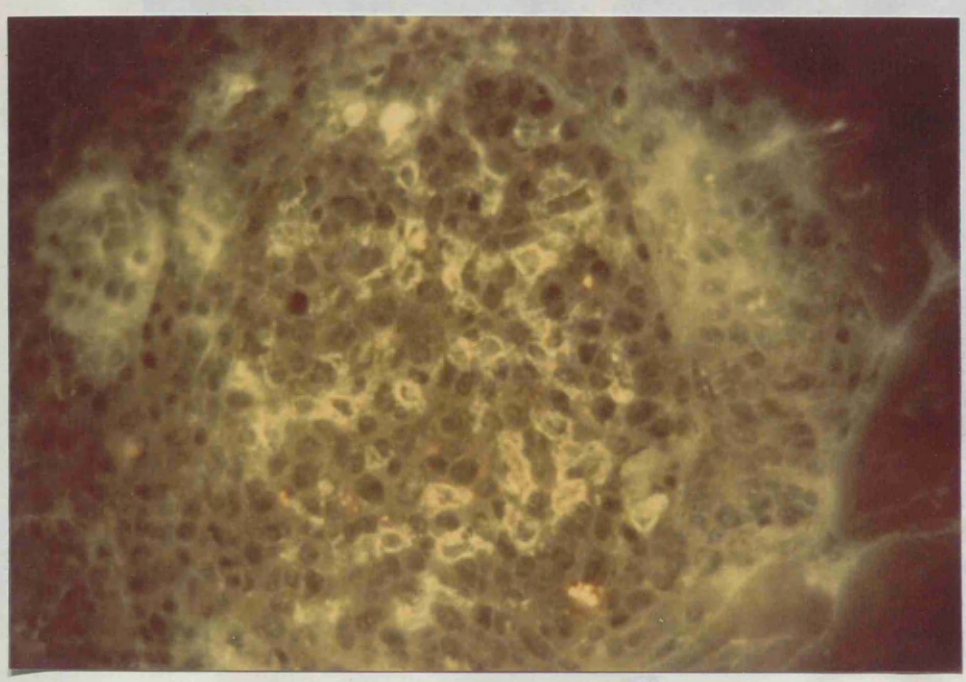


Fig. 1.2.t.

Fluorescence micrograph. Frozen section of chicken breast muscle 35 days after injection of Freund's incomplete adjuvant (FIA) containing human serum albumin as added antigen. Section stained with FITC-sheep anti-chicken 7S globulin. The staining reveals 7S immunoglobulin on the surface of dendritic cells within a germinal centre in the granuloma. Unaffected muscle fibres can be seen at bottom right.

x 720

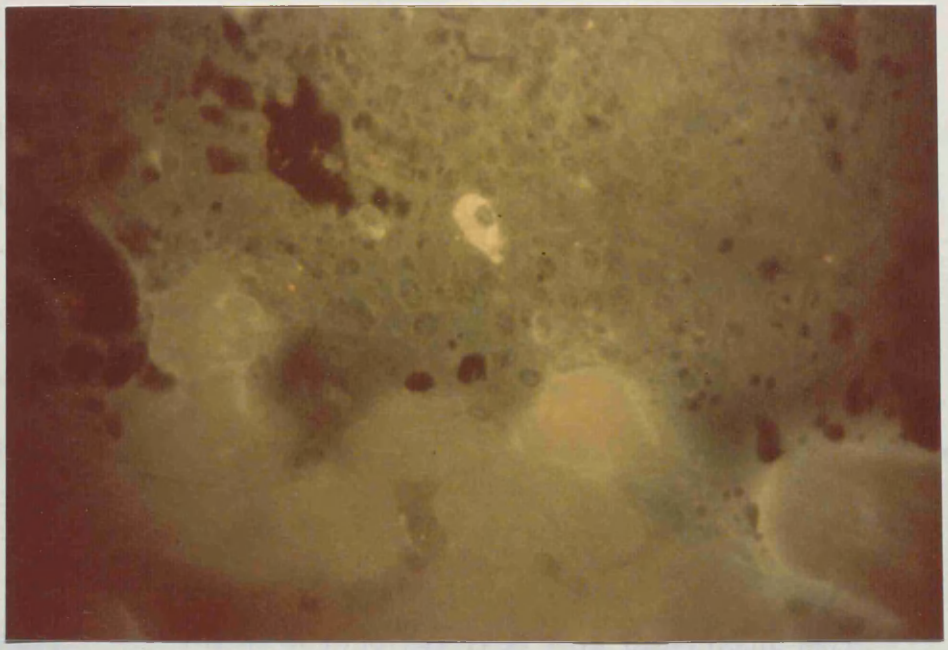


Fig. 1.2.u.

Fluorescence micrograph of FCA granuloma one week after local injection. Frozen section stained by the sandwich method to reveal antibody to HSA. Seen here at the interface between intact muscle fibres and granulomatous tissue is a mature anti-HSA producing plasma cell.

x720

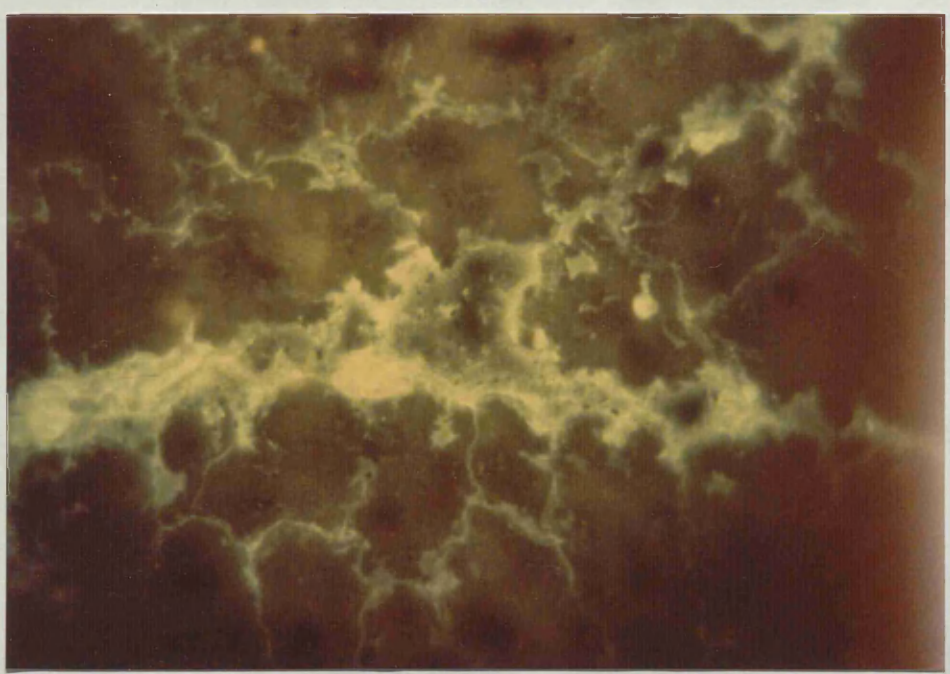


Fig. 1.2.v.

Fluorescence micrograph of FCA granuloma 4 weeks after local injection in the breast muscle of a chicken. Frozen section stained with anti chicken-7S immunoglobulin. All channels between muscle fibres are saturated with globulin.

x720

sites no plasma cells were observed. Moderate amounts of anti-HSA globulin and 7S globulin were seen in the intercellular spaces of the granuloma and surrounding tissue but in no way comparable to the corresponding FCA reaction. On the other hand easily visible with anti-7S staining at this time in both the spleen and granuloma were large well demarcated germinal centres with a striking dendritic cell surface staining pattern (Fig.1.2.t.). This contrasts with the FCA reaction in which no centres were seen in the granuloma.

Summary: The main findings from this experiment were:

1) That after the first week, except for occasional "antibody centres", the spleen did not take part in antibody production to the antigen HSA included in the adjuvant emulsion.

2) The granuloma becomes increasingly saturated with antibody and 7S-immunoglobulin.

3) While germinal centres were seen in the spleen of both FIA and FCA animals and in the local granuloma of the FIA group throughout this time period, none were observed after one week in the FCA granuloma.

The facts support the original contention by French et al., (1970) that the granuloma is actively producing specific antibody at the time when the adjuvant effect is most pronounced, i.e. after four weeks. The exact significance of the incidence of germinal centre formation in these lesions is not clear.

2. AGE DEPENDENCE OF ADJUVANCE

In order to test the possibility that the adjuvant effects on serum antibody levels observed in chickens injected with Freund's complete and incomplete emulsions might be partly due to age, the following experiment was designed.

Both a young (6 week old) and adult (12 - 18 week old) group of chickens were injected with either HSA in FCA or FIA and serum antibody levels samples plotted over a period of four weeks. The levels of antibody to HSA in adult birds was compared to that of young chickens. The results are expressed in Fig 2.1 & Table . As can be seen from the graph there is no substantial difference between adult and young birds in their response to Freund's adjuvant. As seen previously in section 1.2.q , the FCA produced an initial rise in antibody with a peak about day 10 roughly the same for both adult and young groups. This was followed by a decline in antibody level to the 14th day, which was more pronounced in the young birds, followed by a logarithmic increase in antibody levels to as far as the 42nd day. From the 22nd day onwards there is no discernible difference in the antibody levels between the two groups.

The FIA groups were broadly similar. An initial peak at day 10 was followed by a sharp decline in young birds and a more gentle decline in adult birds, to the 14th day. At this point there is some difference. The antibody level of serum from the young birds begins to climb after the 14th day so that by day 35 the level is of the same order as the initial peak. After this a second decline takes place to day 42. This is in contrast to the adult FIA group. The

TABLE 2.1.

COMPARISON OF SERUM ANTIBODY RESPONSES OF YOUNG AND ADULT BIRDS AFTER A SINGLE INJECTION OF HSA IN FREUND'S COMPLETE OR INCOMPLETE ADJUVANT.

BIRD NO.	TREATMENT	AGE	SERUM ANTIBODY LEVEL AT DAY							
			7	10	12	14	22	28	35	42
75070	FCA 40µg HSA + 2mg M.avium in w/o	ADULT (12 weeks)	0.3	6.7	7.1	4.1	6.2	118.1	279.2	220.2
75071			1.5	30.6	32.9	27.4	11.7	52.8	-	-
75072			0.1	4.3	14.1	8.4	7.7	13.5	46.2	269.7
75073			6.3	33.4	23.9	19.9	43.5	94.1	-	-
			2.05	18.75	19.5	14.98	17.28	69.63	162.7	237.4
75082	FIA 40µg HSA in w/o	ADULT (12 weeks)	0.1	0.5	4.8	5.3	1.5	0.8	0.8	1.0
75083			8.8	25.3	17.0	17.3	6.6	6.7	5.3	2.8
75084			7.6	13.4	14.3	12.6	24.4	49.6	-	-
75085			5.3	10.3	5.0	3.0	2.7	5.8	-	-
			5.45	12.38	10.28	9.55	8.80	15.73	3.05	1.9
75086	FCA 40µg HSA + 2mg M.avium in w/o	YOUNG (6 weeks)	2.3	19.7	11.4	7.5	64.5	90.5	136.6	112.7
75087			4.0	12.3	4.7	2.8	20.8	193.5	297.9	297.3
75088			3.3	7.3	4.0	3.5	17.9	56.5	103.4	211.3
75089			1.4	12.6	8.6	6.5	8.2	49.5	66.8	68.4
75090			3.9	13.5	4.9	2.7	9.9	21.2	42.2	62.0
			2.98	13.1	6.7	4.6	24.3	82.2	129.4	150.3
75091	FIA 40µg HSA in w/o	YOUNG (6 weeks)	5.2	1.7	1.1	0.4	3.0	8.2	13.2	7.8
75092			0.3	0.2	0.4	0.4	1.7	3.6	5.3	2.8
75093			0.7	7.4	3.6	2.9	3.6	16.8	14.3	8.4
75094			10.9	9.2	4.7	4.2	3.2	6.2	10.7	9.2
75095			6.8	11.3	5.7	4.2	6.4	10.9	15.1	4.3
			4.8	5.9	3.1	2.4	3.6	9.1	11.7	6.5

Fig. 2.1.

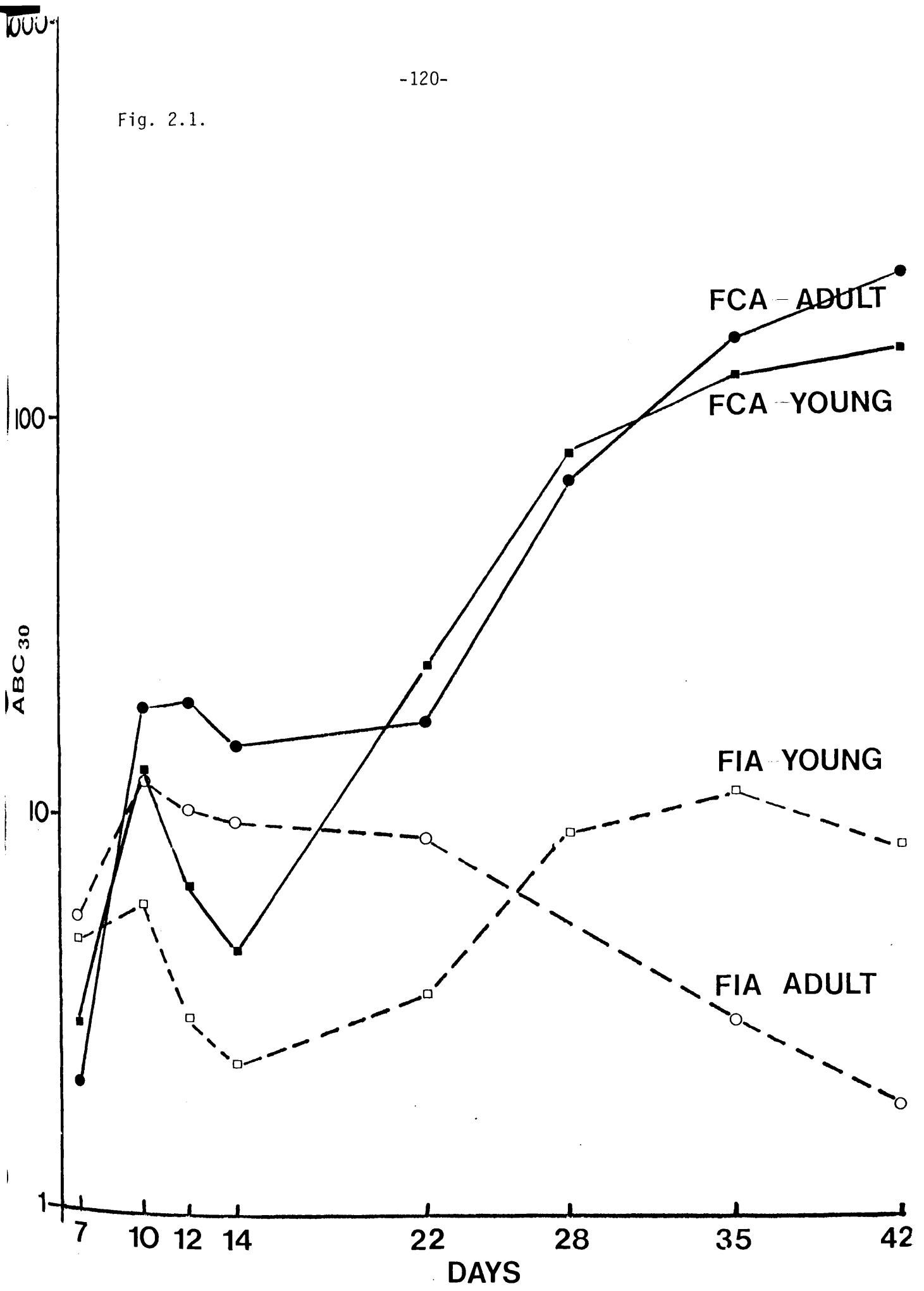
Comparison of the circulating antibody responses of young and adult birds after the intramuscular injection of 40μg HSA in Freund's complete and incomplete adjuvant.

(●) adult birds - FCA (■) young birds - FCA

(○) adult birds - FIA (□) young birds - FIA

Each point represents the mean value of a group of either four (adult) or five (young) birds.

Fig. 2.1.


1000
100
10
1
ABC30
FCA ADULT
FCA YOUNG
FIA YOUNG
FIA ADULT
7
10
12
14
22
28
35
42
DAYS

initial peak at day 10 is followed by a long gradual decline to the 42nd day.

The reasons for the appearance of a second peak in antibody production in the young FIA birds is not clear but may be due to the continued supply of bursal cells to the lymphocyte pool. The adult birds no longer have a bursa at this age.

3. EFFECT OF DOSE OF MYCOBACTERIAL COMPONENT OF FREUND'S ADJUVANT ON SERUM ANTIBODY LEVELS TO AN INCORPORATED ANTIGEN (HSA)

The experiments on granuloma formation carried out in this laboratory have normally used a 5 mgm quantit y of heat-killed mycobacteria added to the oil-phase of Freund's complete adjuvant. This large dose is to a certain extent empirical and it was felt necessary to examine the adjuvant effect of a range of mycobacterial doses. Experiments of this nature have been carried out in other species (Freund et al. 1956) but have not been reported in the chicken. Lurie (1931) in the rabbit, showed that even tiny quantities of mycobacteria were sufficient to ensure a powerful delayed response to tuberculin.

One of the ever present aims behind work of this kind is the use of adjuvants in man to boost immune responses against a number of agents, in large scale immunisation schedules. BCG organisms have achieved widespread use in the treatment of certain forms of cancer (Mathe 1971). It is therefore essential to know the optimum dose of organisms, heat-killed or living, extracted or non-extracted, which will achieve the desired adjuvant effect with the least harmful side results.

Using the chicken breast granuloma as a model the dose of mycobacteria added to the oil vehicle of Freund's complete adjuvant was varied from 50 µgm to 5 mgm, and the adjuvant emulsion made up in the usual way. Groups of birds were injected with these emulsions containing added antigen in the form of human serum albumin (HSA) and blood samples taken over a period of several weeks. The formation and development of a granuloma at the site of injection in the breast was monitored by palp ation with the fingers. After a period of several weeks the animals were killed, the granulomatous lesions examined in situ and histologically, on tissue sections. The granulomata were extracted for antibody and these extracts, along with collected blood samples, tested for antibody levels by the Farr technique.

The results are shown in Fig. 3.1. As a control one group of animals received FIA containing antigen. As can be seen from the graph there is virtually no difference in response to the antigen between the various groups of FCA animals. (It appears therefore that 50 µgm of included mycobacteria incorporated into FCA is as effective as 5 mgm.) After an initial peak at day 10 - 12 the antibody levels declined slightly to day 14. From day 14 to day 22 there was a refractory period followed by a very sharp rise in antibody levels from day 22 to day 28 with a second later plateau from day 24 onwards. The FIA group responded with an initial peak at day 10 comparable to the FCA groups but this peak was followed by a gradual decline to day 42. The highest antibody levels were obtained from the lowest dose of mycobacteria (50 µgm) but this was not found to be statistically significant.

TABLE 3.1.

EFFECT OF DOSE OF MYCOBACTERIA INCORPORATED IN FREUND'S COMPLETE ADJUVANT ON SERUM ANTIBODY LEVELS TO AN ADDED ANTIGEN (HSA).

BIRD NO.	TREATMENT	ANTIBODY LEVEL AT DAY							
		7	10	12	14	22	28	35	42
75107	FCA	0.2	8.1	10.2	9.5	4.6	4.2	5.6	3.2
75108	5mg M.avium	0.5	129.9	118.2	40.9	12.9	112.4	229.8	176.5
75109	40µg HSA in	0.4	27.7	43.2	28.3	51.5	236.9	229.4	296.2
75110	w/o emulsion	0.3	-	7.0	4.7	5.6	164.7	225.1	180.4
	mean	0.4	55.2	44.7	20.8	18.7	129.6	172.5	164.1
75070		0.3	6.7	7.1	4.1	6.2	118.1	279.2	220.2
75071	2mg M.avium	1.5	30.6	32.9	27.4	11.7	52.8	-	-
75072	40µg HSA in	0.1	4.3	14.1	8.4	7.7	13.5	46.2	254.5
75073	w/o emulsion	6.3	33.4	23.9	19.9	43.5	94.1	-	-
	mean	2.1	18.8	19.5	14.9	17.3	69.6	162.7	138.4
75111		0.4	19.3	64.3	52.5	46.9	40.0	79.7	123.8
75112	1mg M.avium	1.8	19.9	14.0	19.0	11.7	17.4	56.7	110.3
75113	40µg HSA in	2.2	12.3	10.5	9.9	1.8	18.8	178.1	271.8
75114	w/o emulsion	1.4	2.8	7.7	10.4	37.6	112.9	172.9	153.6
	mean	1.5	13.6	24.1	22.9	24.5	47.3	121.9	164.8
75115		0.7	20.8	15.0	12.0	33.3	48.4	107.4	283.2
75116	500µg M.avium	4.3	12.1	7.7	4.8	12.8	38.4	62.2	67.9
75117	40µg HSA in	0.3	62.3	196.9	99.9	162.8	368.1	206.2	418.6
75118	w/o emulsion	2.3	5.9	7.3	7.7	6.8	25.4	135.1	250.9
	mean	1.9	25.3	56.7	28.4	53.9	120.1	127.7	255.2
75119		6.5	45.3	19.1	10.6	19.7	65.4	118.4	140.2
75120	250µg M.avium	0.2	4.3	2.5	1.9	9.6	50.2	113.3	46.1
75121	40µg HSA in	4.9	5.8	5.8	7.0	8.9	261.6	-	-
75122	w/o emulsion	2.0	6.0	6.6	8.6	9.2	43.5	153.8	118.9
	mean	2.9	15.4	8.5	7.0	11.9	105.2	128.5	101.7
75123		0.3	2.9	12.0	20.2	20.3	202.1	-	377.5
75124	50µg M.avium	0.3	14.7	23.6	19.7	52.0	234.6	299.1	443.4
75125	40µg HSA in	0.4	0.7	2.4	6.3	21.8	292.1	365.7	404.7
75126	w/o emulsion	0.1	0.9	2.7	3.5	3.0	16.7	41.5	39.4
	mean	0.3	3.9	10.2	12.4	24.3	186.4	235.4	316.3
75082	FIA	0.1	0.5	4.8	5.3	1.5	0.8	0.8	1.0
75083		8.8	25.3	17.0	17.3	6.6	6.7	5.3	2.8
75084	40µg HSA in	7.6	13.4	14.3	12.6	24.4	49.6	-	-
75085	w/o emulsion	5.3	10.3	5.0	3.0	2.7	5.8	-	-
	mean	5.5	12.4	10.3	9.5	8.8	15.73	3.1	1.9

Fig. 3.1.

The effect of the dose of mycobacteria incorporated in Freund's complete adjuvant on the circulating antibody response of chickens to an intramuscular injection of 40µg HSA given in the adjuvant

(□) 5 mg M. avium

(■) 2 mg M. avium

(▲) 1 mg M. avium

(▽) 500 µg M. avium

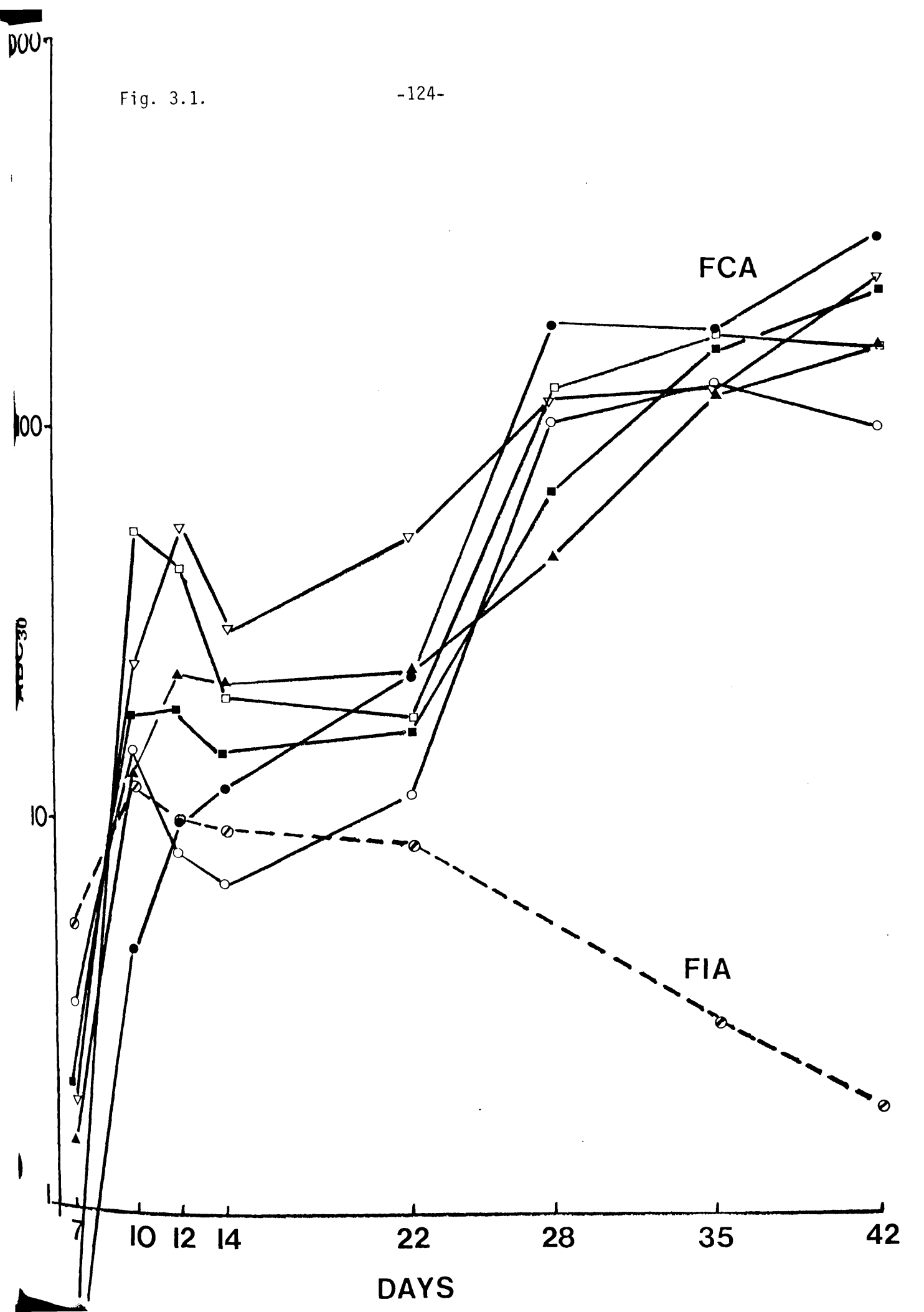
(○) 250 µg M. avium

(●) 50 µg M. avium

(⊘) none i.e. FIA

Each point represents the mean value of a group of four animals.

Fig. 3.1.



Extracts from granulomata

There was no histological difference in the granulomata from the various groups. The antibody levels from the extracted tissues were very different. There was an obvious inverse relationship between the dose of M. avium and level of antibody extracted. This is shown in Fig. & Table 3.2. The abscissa represents the ABC_{30} antigen binding capacity of the granuloma extract and the ordinate the doses of M. avium in logarithmic form. At 50 µgms the ABC_{30} is of the order of 500 µg/ml while at 5 mgm it is below 100 µg/ml. This suprising result must have a bearing on the use of these adjuvants in antibody production and possibly in immunotherapy as well. It suggests that the required amount of the active component of the adjuvant is very small, 50 µg or less, and an increase in the number of mycobacteria does little to increase the resulting circulating antibody levels. An increase in the weight of mycobacteria may even reduce the effectiveness of the adjuvant at the site of antibody production, i.e. the granuloma.

4. EFFECT OF SEPARATION OF COMPONENTS OF ADJUVANT EMULSIONS ON SUBSEQUENT ANTIBODY PRODUCTION.

White (1973) described an experiment in which the added antigenic (HSA) component and the mycobacterial component of Freund's complete adjuvant were separated in time, the bacteria being given intramuscularly in mineral oil four days after the intramuscular injection at the same site of HSA in a water-in-oil emulsion. The adjuvant effect of this separated mixture proved as effective on the late antibody response as the combination of HSA and mycobacteria in the water-in-oil

TABLE 3.2.

EFFECT OF DOSE OF MYCOBACTERIAL COMPONENT OF FREUND'S COMPLETE ADJUVANT ON AMOUNT OF ANTIBODY EXTRACTED FROM GRANULOMATA SIX WEEKS AFTER I.M. INJECTION OF HSA IN ADJUVANT.

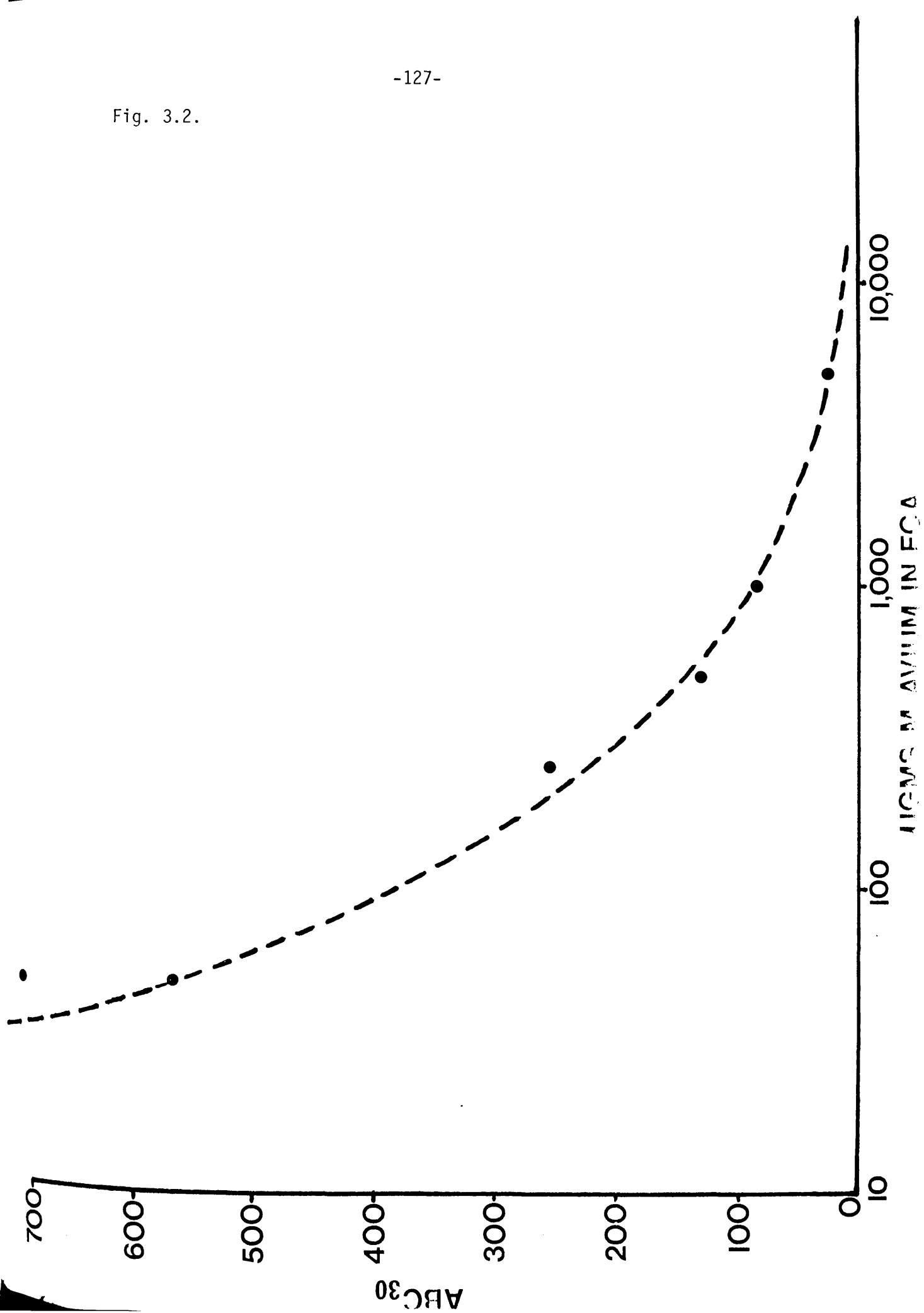
BIRD NO.	DOSE OF M.AVIUM	ANTIBODY EXTRACTED PER GM OF TISSUE
75107	5 mg	35
75108	"	30.7
75109	"	6.5
75110	"	25.4
		24 MEAN
75111	1 mg	69.9
75112	"	83.2
75113	"	189.5
75114	"	4.4
		87 MEAN
75115	500 µg	252.0
75116	"	38.9
75117	"	124.0
75118	"	105.4
		130 MEAN
75119	250 µg	230.2
75120	"	454.2
75121	"	148.0
75122	"	188.1
		255 MEAN
75123	50 µg	567.2
75124	"	687.6
75125	"	1326.7
75126	"	259.5
		710 MEAN

Fig. 3.2.

Effect of dose of mycobacteria incorporated in Freund's complete adjuvant on the amount of antibody extracted (per gm of tissue) at six weeks from granulomata of birds injected intramuscularly with 40μg HSA in the adjuvant emulsion.

Each point (●) represents the mean value of a group of four animals.

Fig. 3.2.



injection. It was suggested that the mycobacteria need to be in the tissues at the time when germinal centres are forming.

These observations raised a number of questions, concerning the constituent components of Freunds adjuvant and their presentation to the animal. These questions were:

1. Does the HSA have to be in emulsion form of water-in-oil?
2. Does the mycobacterial component have to be in water-in-oil form?
3. Can the W/O be replaced by oil or saline?
4. Do the antigen, oil, and mycobacteria have to be given together?

Accordingly an experiment was set up with 7 groups of chickens (5 per group). The first group received 100 µg HSA in aluminium phosphate at day 0, followed by 5 mg M. avium in water-in-oil. The second group were injected with 100 µg HSA in saline at day 0, followed by 5 mg M. avium in W/O at day 7. The third group received 100 µg HSA in water-in-oil at day 0, followed by 5 mg M. avium in water-in-oil at day 7. Group 4 were injected at day 0 with 100 µg HSA in water-in-oil, followed by 5 mg M. avium at day 7 in an oil-in-water mixture. The fifth group received 100 µg HSA in W/O followed by 5 mgm M. avium at day 7 in saline. Group 6 received 100 µg HSA in water-in-oil at day 0 plus 5 mgm M. avium at day 7 in pure mineral oil. Finally as control, group 7 received no HSA and 5 mg M. avium in mineral oil at day 7.

Blood samples were collected from the animals during the course of the experiment and the serum tested for antibody to HSA by the Farr technique. The granulomata resulting from these injections were examined by palpitation during the experiment and at post-mortem.

TABLE 4.1.

EFFECT OF SEPARATION OF COMPONENTS OF FREUND'S ADJUVANT EMULSIONS ON SUBSEQUENT SERUM ANTIBODY LEVELS.

BIRD NO.	GROUP	TREATMENT		ANTIBODY LEVEL (ABC_{30}) ON DAY :-							
		DAY 0	DAY 7	5	7	11	14	21	29	35	42
75367		100µg HSA	5mg M.avium	8.96	14.79	11.01	7.23	1.12	0.69	0.13	0.70
374	A			1.58	29.75	14.64	6.53	1.02	0.58	1.79	2.41
381		in AlPO4	in w/o	11.12	19.97	10.26	10.23	1.57	1.90	2.47	2.20
			MEAN	7.22	21.50	11.97	7.96	1.23	1.056	1.46	1.77
75368		100µg HSA	5mg M.avium	3.31	23.40	23.81	13.06	3.48	2.35	0.97	1.55
375	B			4.64	11.06	9.52	1.93	0.41	-	-	-
382		in saline	in w/o	3.71	11.51	6.49	1.14	4.13	0.64	0.67	0.46
			MEAN	3.88	15.32	13.27	5.37	2.67	1.49	0.82	1.05
75369		100µg HSA	5mg M.avium	2.85	12.89	10.77	6.76	4.00	10.82	13.63	14.39
376	C			4.99	22.18	69.12	26.40	9.15	144.75	166.59	194.05
383		in w/o	in w/o	18.65	45.53	33.64	46.84	22.04	90.24	138.31	99.08
			MEAN	8.83	26.86	37.84	26.66	11.73	81.93	106.17	102.50
75370		100µg HSA	5mg M.avium	3.85	12.91	18.97	16.45	2.59	4.20	17.85	39.92
377	D			4.68	9.49	19.07	11.59	15.11	200.31	175.29	180.17
384		in w/o	in o/w	5.74	46.48	16.98	7.91	4.55	9.72	44.20	23.04
			MEAN	4.75	22.96	18.34	11.98	7.41	71.41	79.11	81.04
75371		100µg HSA	5mg M.avium	1.78	5.04	8.06	9.79	10.81	14.39	7.88	13.20
378	E			3.33	26.00	21.33	13.86	21.54	107.42	130.98	99.60
385		in w/o	in saline	13.28	25.45	16.35	14.41	14.09	15.52	43.91	30.10
			MEAN	6.13	18.83	15.24	12.68	15.48	54.77	60.92	47.63
75372		100µg	5mg M.avium	0.21	10.39	6.54	5.62	11.82	22.54	19.71	23.37
379	F			8.36	7.39	27.75	37.73	19.65	39.05	80.24	74.08
386		in w/o	in Drakeol	4.11	10.86	24.63	11.60	14.35	22.77	149.53	123.08
			MEAN	4.22	9.54	19.64	18.31	15.27	27.88	83.16	73.51
75373		-	5mg M.avium	0.09	0.32	0	0	0.10	0.08	0.10	0.41
380	G			0.47	0.20	0.25	0.43	0.51	0.47	0.14	0.07
387			in w/o	0.91	0.90	0.90	0.99	0.55	0.98	0.64	0.73
			MEAN	0.49	0.47	0.38	0.47	0.38	0.51	0.29	0.40

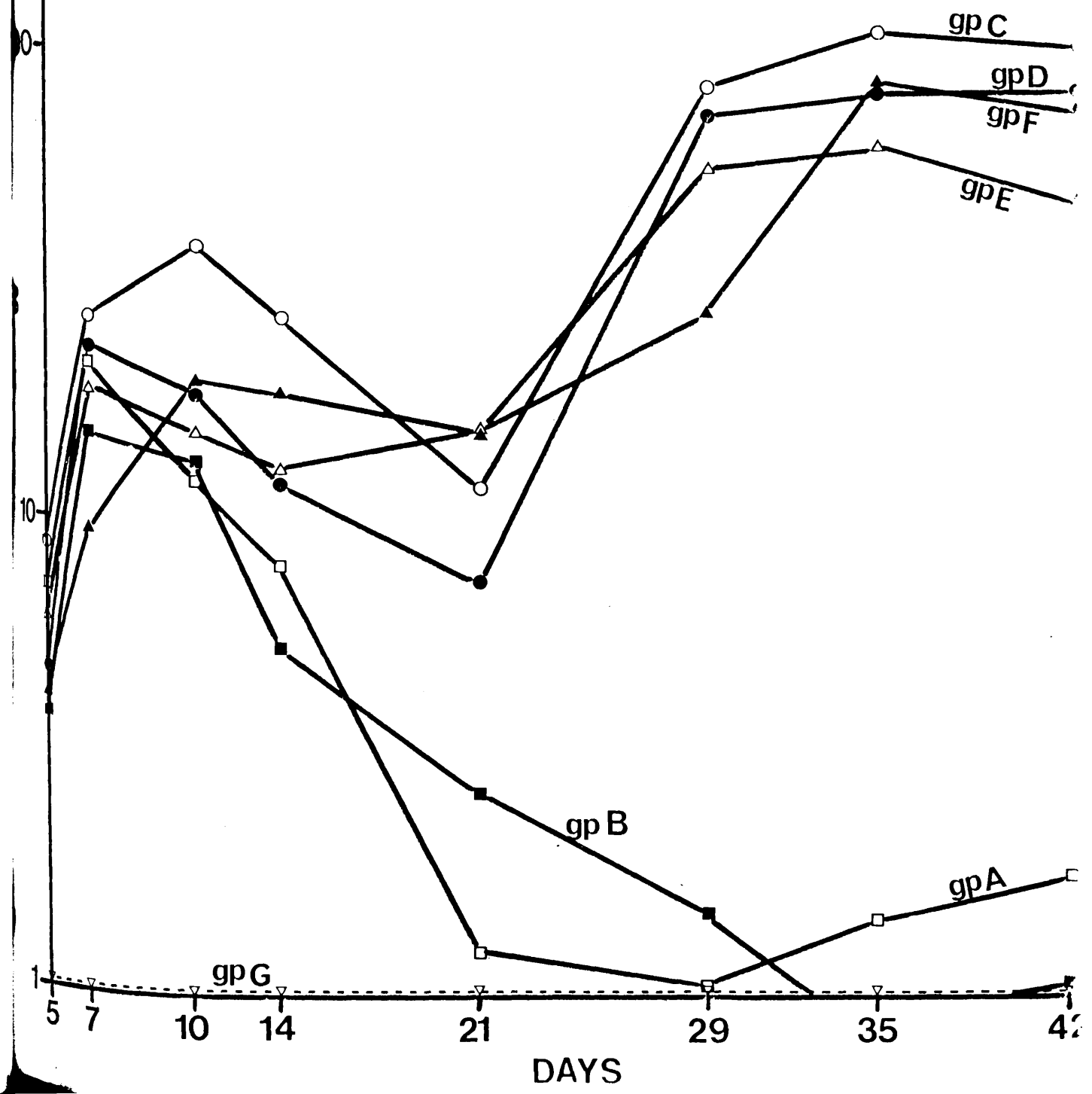
Fig. 4.1.

The effect of separation of the components of Freund's complete adjuvant (FCA) on the circulating antibody response of adult chickens to an intramuscular injection of 40µg HSA incorporated in the following vehicles:-

Symbol	group	day 0	day 7
(□)	A	100µg HSA in AlPO4	5mg M. avium in w/o
(■)	B	100µg HSA in saline	5mg M. avium in w/o
(○)	C	100µg HSA in w/o	5mg M. avium in w/o
(●)	D	100µg HSA in w/o	5mg M. avium in o/w
(△)	E	100µg HSA in w/o	5mg M. avium in saline
(▲)	F	100µg HSA in w/o	5mg M. avium in Drakeol
(▽)	G	-	5mg M. avium in Drakeol

Each point represents the mean value of a group of three birds.

SYMBOL	GROUP	DAY 0	DAY 7
□	A	100µg HSA (0.1ml) + $AlPO_4$ (0.9ml)	5mg M.avium w/o (0.45ml)
■	B	100µg HSA saline 0.5ml	5mg M.avium w/o (0.45ml)
○	C	100µg HSA w/o 0.45ml	5mg M.avium w/o (0.45ml)
●	D	100µg HSA w/o 0.45ml	5mg M.avium o/w (o.45ml)
△	E	100µg HSA w/o 0.45ml	5mg M.avium saline (0.45ml)
▲	F	100µg HSA w/o 0.45ml	5mg M.avium Drakeol (0.45ml)
▽	G	-	5mg M.avium Drakeol (0.45ml)



The different vehicles resulted in different types of granuloma though this is not shown in Table 4.1. Groups A, B, C, D, F, G, had granulomas typical of FCA injections. The granulomata in group E were smaller and far more localised than the other groups.

The antibody levels to HSA in the different groups is expressed graphically in Fig.4.1 . As can be seen clearly from this graph, groups C, D, F, and E have responded in a similar fashion, groups A and B, though strikingly different to the first four groups, are similar to each other, and group G, the control, showed no antibody production to HSA.

Three important points emerge from this graph. Firstly it seems necessary to incorporate the antigen (HSA) in the water-in-oil vehicle, as neither the antigen in Al PO_4, nor the antigen in saline have resulted in the late adjuvant response to HSA, typical of the FCA reaction. Why this should be so is not clear. The association of the HSA with Al PO_4 should result in the formation of an antigen depot at the site of injection, and Al PO_4 is commonly used as an adjuvant because of this. The HSA in saline has resulted in a good primary response but no secondary delayed antibody peak. Presumably the antigen has disappeared from the site prior to the arrival of mycobacteria.

Secondly there does not seem to be a requirement for the mycobacteria to be incorporated in an oil vehicle in order to achieve an adjuvant effect. This is amply demonstrated in group E. The granulomas resulting from this mixture were smaller and more contained than the others but the antibody response was similar.

Thirdly, as shown by White (1973) the two components do not have

to be injected simultaneously. In this experiment the interval between antigen and mycobacteria has been extended to 7 days, without affecting the adjuvant response.

It seems as if the major requirements for the adjuvant to work are:

1. A slow release of antigen from an oily depot.
2. The presence of mycobacteria during the first week at the antigen depot site.

5. Homing of labelled thymus and bursa cells to granulomata induced by HSA in FCA or FIA in the chicken.

It seems clear from the preceding experiments and from previous studies (French et al., 1970) that the bulk of the antibody formed during the second or delayed peak after injection of antigen in Freund's complete adjuvant is derived from the granuloma and not the spleen. The origin of the antibody-producing B cells in the granuloma is unknown though it is possible that they or their precursors are derived from immature bursal (B) cells in the circulation, which are trapped at the site of the granuloma under the influence of antigen.

Similarly, little is known about the contribution of thymus-derived (T) cells in the development of the granuloma though earlier studies (White, 1971) demonstrated that neonatal thymectomy and irradiation completely abolished the FCA granuloma in chickens.

To investigate these two problems birds bearing FCA or FIA granulomata of various ages (from 1-4 weeks) were given an intravenous infusion of autologous, tritiated-adenosine-labelled thymus or bursa cells, killed exactly 24 hours later, and sections of the spleens and granulomata processed for

autoradiography. Considerable difficulties were encountered in quantitation of the labelled cells, due mainly to lack of uniformity of granuloma sections.

a) Thymus cells

Very few labelled thymus cells were observed at any time in the FIA granulomata, and the majority of cells counted were in the spleen. In contrast the FCA granulomata contained variable numbers of thymus cells (Figs. 5.2, 3, and 4) with the highest number at one week (approximately 60% of the total number counted in spleen and granuloma)dropping to about 25% at four weeks. At this later time the labelled cells were seen mainly in the lymphoid areas of the granuloma while at one week the majority of cells were situated in or around the numerous small blood vessels which characterise the early FCA lesions.

b) Bursa cells

Few labelled bursa cells were seen at any time in either the FCA or FIA granulomata with slightly higher numbers in the FCA animals (Fig. 5.1.). Most of the cells counted were in the spleen. A possible explanation for this paucity of labelled bursa cells is the considerable size of the FCA granuloma, which is up to 20 times the size of the spleen, so that there is a dilution of the number of cells counted. This is unlikely in view of the thymus cell results.

This experiment suggests that large numbers of thymus cells appear early in the developing FCA granulomas but that this number drops down by the fourth week. In contrast bursa cells do not appear to make an extensive contribution to these granulomata. A likely explanation of these results is that proposed by White (1973) who suggested that the origin of the antibody-producing B cells in this type of lesion was a pool of cells associated with the germinal centres of the spleen i.e. the progeny of bursa cells which have entered germinal centres and divided in them under the influence of antigen upon the surface of nearby dendritic cells.

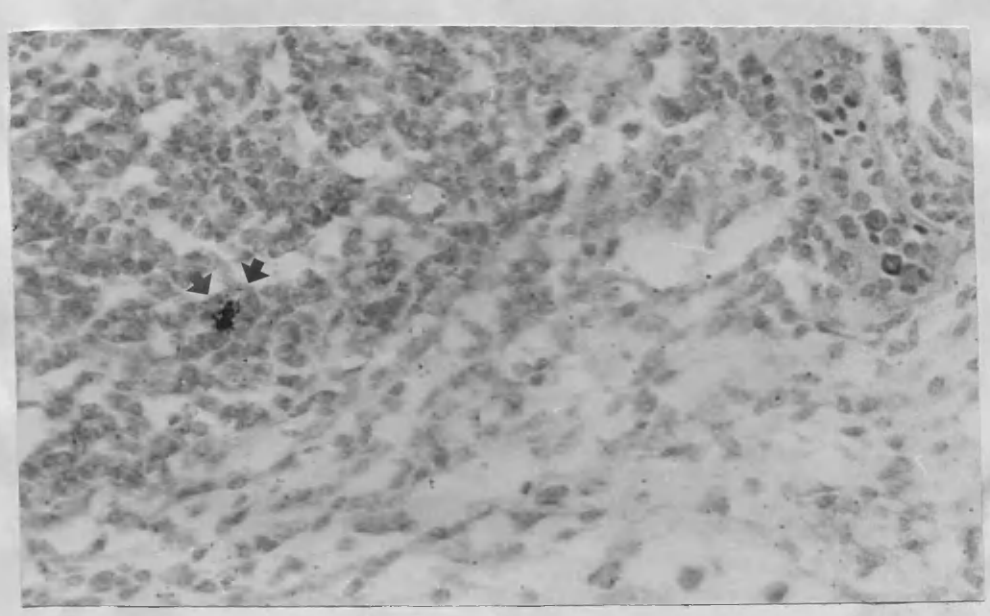


Fig.5.1. Autoradiograph of a labelled bursa cell (arrowed) in a lymphoid nodule of a four week-old FCA granuloma. This bird had received 24 hours previously an intravenous injection of 5 x 10^7, ^{3}H-adenosine-labelled autologous bursa cells. To the right of the micrograph is a small blood vessel packed with erythrocytes and mononuclear cells.

U.P. x 1200

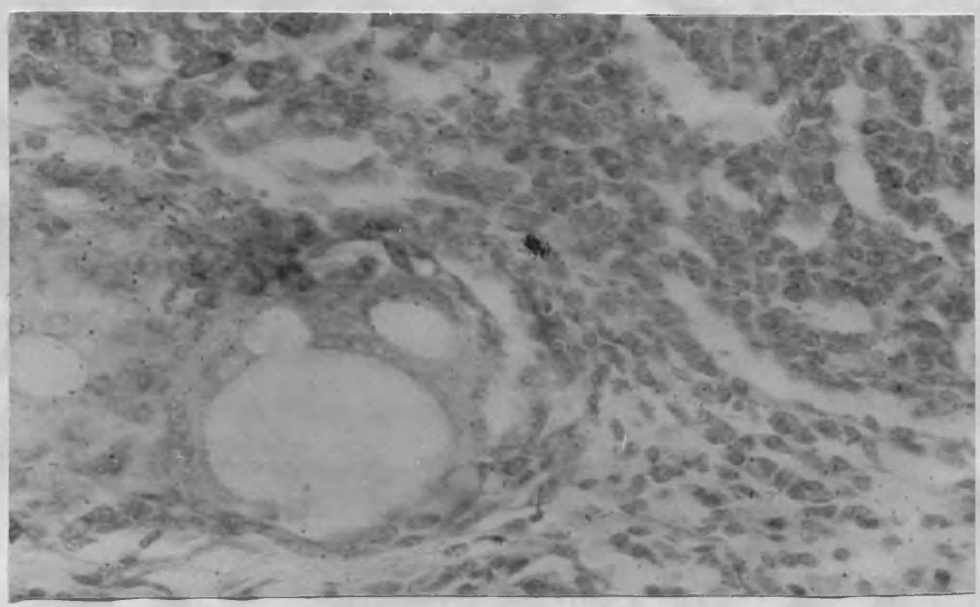


Fig. 5.2. Similar lesion after infusion of labelled thymus cells. A labelled cell is visible at the periphery of a large oil droplet.

U.P. x 1200

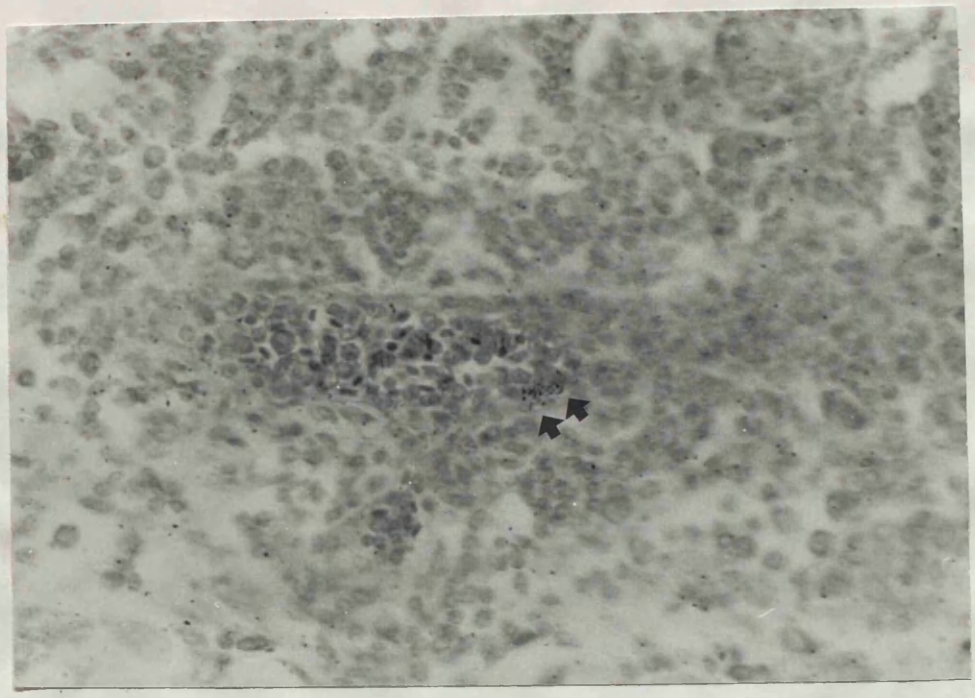
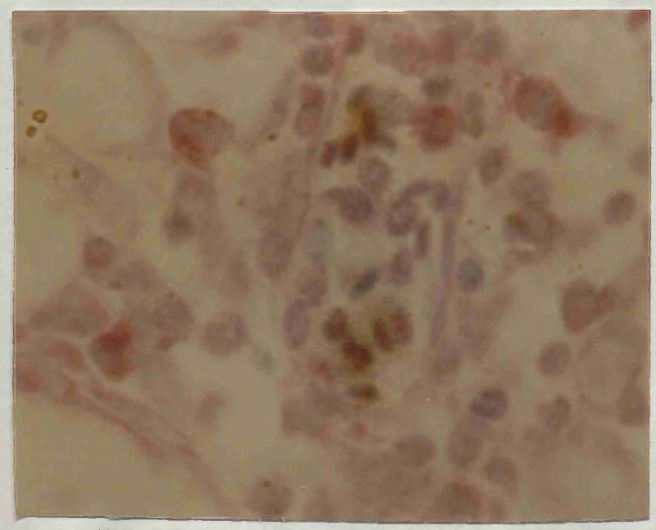


Fig. 5.3. Reactive venule in a lymphoid area of a four week old FCA granuloma. The lumen is packed with small mononuclear cells. This bird had been injected 24 hours previously with 5 x 10^7, ^{3}H-adenosine labelled, autologous thymus cells, two of which can be seen (arrowed) beyond the endothelial cell wall. U.P. x 1200

(a).



(b).

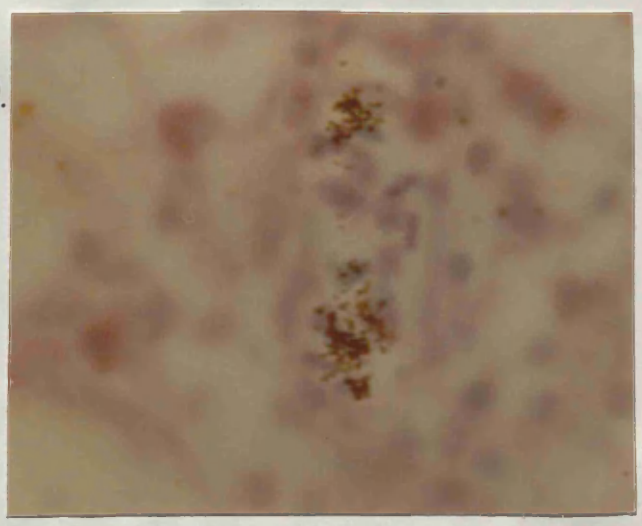


Fig. 5.4. Small blood vessel in a one week-old FCA granuloma in a bird injected 24 hours previously with 5 x 10^7, ^{3}H-adenosine labelled thymus cells. Fig.a: focused on cell layer showing a mixture of erythrocytes and mononuclear cells in the vessel lumen. Fig.b: focused above the cell layer showing that several of the mononuclear cells are from the pool of injected labelled thymus cells. U.P. x 1800

Part II Further analysis of germinal centres

The structure and development of the granulomas which form at the site of injections of water-in-oil emulsion adjuvants has been discussed in the first part of this thesis. It has been shown that Freund's incomplete adjuvant (antigen in water-in-oil) invariably resulted in a local foreign body granuloma characterised by macrophage and lymphocyte infiltration with extensive germinal centre formation. In comparison Freund's complete adjuvant (antigen in water-in-oil plus added mycobacteria) resulted in the formation of a massive complex granuloma with necrotic tubercular lesions surrounded by giant cells, epithelioid cells, plasma cells, small lymphocytes, and heterophils. In direct contrast to the incomplete adjuvant few, if any, local germinal centres could be seen. Occasional bizarre pyroninophilic lymphoid cell accumulations resembling germinal centres were occasionally seen though lacking the recognisable compact structure of the typical germinal centre. Often these aggregates had small epithelioid granulomas at their periphery or even within their boundaries.

It has been suggested (White, 1973) that the high antibody levels which result from administration of FCA are due to an effect on germinal centre formation, acting to disrupt the normal feedback mechanism of antibody production. In view of the observed contrast between the many germinal centres of FIA granulomas and the lack of these structures in the FCA granulomas it was decided to attempt a further analysis of germinal centre function in the chicken.

Germinal centres: structure and function in the immune response

Since the early descriptive reports of Flemming (1885) and subsequently of Hellman (1921), Maximow (1927), and Conway(1937) lymphatic tissue germinal centres have been a subject of considerable interest to the anatomist, pathologist, and immunologist alike. This interest derived from the implication of germinal centres in various aspects of the immune response and occurred in two major phases.

The first wave of interest reached its height in the 1930's and centered on the rather vitriolic controversy between Flemming's concept of the keimcentrum "germinal centre" as a site of lymphocytopoeisis and Hellman's idea of this structure as a site of destruction of lymphocytes. Flemming based his theory on the frequency of mitotic figures seen in the germinal centres whereas Hellman, using mainly human material obtained at autopsy, noticed considerable cell debris within the centres and called them "reaction centres". He and his followers stuck to this idea even when its was pointed out that mitotic figures were plainly visible in some of their displayed "reaction centres". Maximow (1927) adopted an intermediate position, noting both the lymphocytopoeitic and cell debris type of centre. Conway (1937) first unified both theories by describing the transition of one type of centre to the other and suggested that Hellman's reaction centre was simply a later stage of Flemming's lymphocytopoeitic centre. She was supported in this approach by Ringertz and Adamson in 1950. These studies are shown in Table IIi

The second wave of interest coincided, not suprisingly, with the

Table II.i. Early studies on germinal centres

also known as "lymphocytopoeitic centres", "secondary lymphoid nodules", "immune bodies", "reaction centres", and "follicle centres".

Year	Author	Finding
1885	Flemming W.	Germinal centre as site of lymphocytopoeisis.
1921	Hellman T.	Reaction centre as centre of lymphocyte destruction.
1927	Maximow A.	Described both types of centre
1937	Conway E.	Suggested progression of Flemming's "germinal centre" to Hellman's "reaction centre" with time.
1950	Ringertz N. and Adamson C.	Detailed analysis of germinal centre formation and structure.

rapid expansion of immunology in the 1960's and 70's, resulting in the current series of International Conferences of Germinal Centres (Nos. 1-6). During this second phase germinal centres have been the subject of intense study and have been implicated in the following aspects of the immune response, shown here in tabulated form.

Table II.2.

1.	Site of expansion of population of immunocompetent cells	- White R.G. (1960) - Good R.A. et al (1969)
2.	Site of generation of B-memory cells	- Thorbecke G.J. (1969) - Buerki et al (1974)
3.	Site of induction of immunological tolerance	- Ada G. and Parish C. (1968)
4.	Site of long-term antigen retention	- Ada G. and Williams J. (1966) - E. and Askonas B. (1968) - White R.G. et al (1970)
5.	Site of antibody production	- White R.G. et al (1970)
6.	Role in feedback control of antibody production	- Sinclair N. et al (1971) - White R.G. (1973)
7.	Homing site for B cells	- Durkin H. et al (1971)
8.	T - B cell interaction site	- Gutman G. and Weissman J. (1971)
9.	Ectopic lymphoid response to inflammatory agents	- Silverstein A.M. and Prendergagast R.A. (1971)

Current Theories Concerning Germinal Centre Function

The first suggestion that the germinal centre was acting as a site of expansion of a population of immunocompetent cells came from White in 1960. This suggestion has since been supported by several other groups notably Good et al (1969) and Thorbecke et al (1965). The basis for this suggestion was the observation that cells produced in rabbit popliteal node germinal centres after a single stimulus of soluble or alum precipitated antigen did not contain detectable antibody. A second antigenic stimulus, however, was capable of converting these "primed" cells to antibody containing cells. He suggested that these "primed" cells were imprinted with a specific capacity to react to a secondary antigenic stimulus.

Later this idea was extended to include the concept of immunological memory (see review by Thorbecke and Lerman, 1976). These authors have defined immunological memory as "... a complex phenomenon which encompasses all changes that lead to an accelerated and enhanced response upon a repeated as compared to a first exposure to a specific antigen." There are two major forms of cellular, immunologically specific memory, corresponding to T and B lymphocytes. T-cell memory is induced within 1 - 2 days after antigen and requires lower antigen doses than B-cell memory. The latter is not detectable until days 3 - 7 and usually requires a higher dose of antigen (Cunningham and Sercatz, 1971).

Considerable support for this concept came from the observation, by various groups, that methods which interfere with germinal centre

formation such as irradiation and drugs also causes loss of 7S memory (Thorbecke, 1969; Grobler et al., 1974).

The germinal centre as a site of long-term antigen retention has been demonstrated by several groups and in several species, using both autoradiographic and immunofluorescent techniques (Ada and Williams, 1966; Unanue and Askonas, 1968; White et al., 1970). Thus White and his colleagues have shown that HSA is still visible in germinal centres as long as 6 weeks after injection. They have argued further that this small fraction of the injected dose of antigen plays a highly significant role in the proliferation of associated lymphoid cells.

The persistance of antigen in germinal centres has been viewed differently by Ada and Parrish (1968). They have suggested that this antigen is well placed to induce a state of immunological tolerance.

Germinal centres have also recently been implicated in the feedback control of antibody production. Sinclair and Chan (1971) proposed that antigen-antibody complexes, bound through the Fc portion of the antibody to dendritic reticular cells in germinal centres act to suppress antibody production to that antigen once a certain circulating threshold of antibody has been reached. They based this proposal on the observation that removal of the Fc portion of passively administered antibody reduced the ability of specific antibody to inhibit the specific immune response by factors of 10 - 1000-fold. White (1973) extended this idea by suggesting that the feedback mechanism is based upon the ability of germinal centres containing antigen-antibody complexes, to net or remove potential antigen-reactive B cells from the circulation until such a time as free antigen has disappeared. He also envisaged

an Fc-dependent antigen-antibody complex localisation on dendritic cells in the germinal centre and suggested that these complexes were responsible for the removal of immuno-competent cells from the circulation. This hypothesis will be discussed in more detail in the following section on avian immunology.

Considerable impetus for many of these proposals came from the demonstration by Durkin and her colleagues (1971) that ^{3}H-adenosine-labelled bursa cells home to germinal centres in the chicken spleen, i.e. that germinal centres are bursa-dependent. This was in contrast to the results of Linna et al (1969) who failed to show germinal centre homing of in situ, ^{3}H-thymidine-labelled bursa cells, but this may have been due to the rapid proliferation of cells in germinal centres with a resultant diminution of isotopic label so that by 48 hours after in situ labelling no cells were seen.

Though Durkin et al (1971) rarely saw transferred labelled thymocytes in germinal centres in the chicken and despite reports that mouse thymus cells do not enter germinal centres (Parrott, 1966), Gutman and Weissman (1971) have suggested, using specific fluorescent antisera that most germinal centre cells in mice are of thymic origin. They further suggested that the germinal centre acts as a site of interaction between T and B cells, with the bone marrow derived cells of the primary follicle reacting directly or indirectly through reticular cells with the germinal centre or secondary follicle thymus-derived cells.

One aspect of germinal centres which is sometimes neglected, but which is nevertheless of considerable importance in understanding their

biological role, is the ability of these structures to form in ectopic i.e. non-lymphoid tissues. Though most studies on germinal centres have been performed on organised lymphoid tissues such as the lymph nodes and spleen, few have been concerned with ectopic germinal centre formation, occurring in, for example, the non-encapsulated lymphoid tissues which line the permeable epithelial surfaces of the body. These include the conjunctiva, the bladder, the ureter, the cervix, or the gut. Other sites of ectopic lympho-follicular inflammation in man which include germinal centre formation are seen in the thyroid, in the deep dermis, in muscle, and in the orbit of the eye (Silverstein and Prendergast, 1971). It appears that lymphocytic infiltration may occur in any tissue of the body, and under certain circumstances, result in organisation into both primary follicles and germinal centres.

Requirements for Germinal Centre Formation

What are the necessary conditions and requirements for germinal centre formation? During the 1960's and early '70's most emphasis was placed on the germinal centre as a bursa-dependent structure, and its major function seen as the production of potential antibody producing cells. This view was based on the demonstration (Cooper et al., 1965) that neonatal bursectomy completely abolishes germinal centre formation and antibody production, and that the majority of cells in chicken germinal centres were shown to be of B-cell origin (Durkin et al., 1971).

An uncomplicated picture of germinal centre requirements a few years ago held that antigen in the form of antigen-antibody complexes

attached to dendritic cells in lymphoid tissues of the body. These cells formed aggregates with the supply of B-lymphocytes available by selecting the B cells specific for the antigen, and this aggregate eventually became recognisable as a germinal centre (White et al., 1970).

Suprisingly therefore it appears that an intact thymus or supply of T cells is also necessary. Nude, thymus-less mice lack germinal centres (De Sousa and Pritchard, 1974; Jacobson et al., 1974; Mitchell et al., 1972) and neonatally thymectomised mice and chickens after local irradiation of the thymus are deficient in their ability to form germinal centres (Hoshi and Mori, 1973; Laissue et al., 1969). Reconstitution of nude mice restores their ability to form germinal centres (De Sousa and Pritchard, 1974; Jacobson and Thorbecke, 1974). It appears therefore that some kind of T-B cell interaction is necessary.

Another limiting requirement which has recently been demonstrated is the level of the C3 component of complement (Pryjama et al., 1974; White et al., 1975). These groups have shown that depletion of C3 through cobra venom interferes with proper germinal centre formation. Pepys (1974) has suggested that it is the macrophage-B cell interaction which is affected by lack of C3.

Finally it has been shown by Toivanan and co-workers (1974), in the chicken that histo-compatible cells are necessary for effective production of germinal centres. Thus histo-incompatible B cells transferred into cyclophosphamide-bursectomised hosts will not restore germinal centre formation, though semi-allogeneic donor cells will do so. It is not clear whether the histocompatibility requirements are for the T-B cell or B-dendritic cell interaction. These requirements are summarised in table II.3.

Table II.3. Requirements for Germinal Centre Formation

1) antigen retained on surface of dendritic cells) as antigen antibody complex
2) specific antibody with intact Fc portion)

3) dendritic cells

4) intact bursa or supply of B cells

5) intact thymus or supply of T cells

6) C_3

7) histocompatibility between T and B or B and dendritic cells

Despite such interest and almost a century of investigation the exact nature of germinal centres, their origin and function, remains unclear. It is not known, for example, whether germinal centres are purely temporary structures formed de novo or more permanent features of lymphoid tissue anatomy. Neither is it known with any certainty what becomes of the progeny of the rapidly dividing cells within germinal centres.

Nevertheless, the presence of these structures in lymphoid and non-lymphoid (ectopic) situations seems to be of great importance in the immune response, indicative perhaps of the degree of sophistication reached in an ongoing response. It is significant therefore that while a FIA granuloma contains many germinal centres few are seen in a FCA

lesion, suggesting perhaps that there is an inhibition of germinal centre formation in the latter situation. In an attempt to examine this possibility a further analysis of germinal centres in the chicken was carried out.

6.1. Structure, Formation and Distribution of Germinal Centres in the Chicken

6.1.1. Structure

The chicken germinal centre is a compact, easily recognisable, unit structure which is widely distributed in the lymphoid tissues. Wherever they occur germinal centres are easily distinguished in stained tissue sections by their compact, circular appearance and by their staining characteristics. The typical germinal centre (Fig. 6.1.1.) is circular or oval in outline, approximately 50 - 200μ in diameter, and clearly delineated from the surrounding lymphoid tissue by a delicate connective tissue capsule. The cells of the germinal centre consist of closely packed, medium sized, slightly basophilic, lymphocytes, including frequent cells in mitosis. The size of the cells and the basophilia are subject to considerable variation, corresponding perhaps to the age of the centres (personal observations). Staining of the germinal centre sections with fluorescein-labelled anti-7S antibody (or antibody to injected antigen), reveals the presence of evenly spaced dendritic cells interspersed with the lymphocytes (Fig. 6.1.2.). This appearance is common to all germinal centres and serves as an easy method for identifying these structures in cryostat tissue sections.

6.1.2. Formation

The sequence of events which leads up to the formation of a germinal centre in the chicken spleen have been described in great

Fig. 6.1.1.

Photomicrograph of a typical germinal centre in a thin section of chicken spleen. Note the circular or oval appearance of the centre and its delineation from the surrounding lymphoid tissue by a delicate connective tissue capsule. The enclosed cells are distinguished by their closely-packed, medium-sized, slightly basophilic appearance.

U.P. x 2000

Fig. 6.1.2.

Fluorescence micrograph of germinal centre in a frozen section of chicken spleen 7 days after the i.v. administration of 10 mgms. HSA. The antigen, in the form of an antigen-antibody complex is seen on the cell-surface and cytoplasmic extensions of evenly spaced dendritic cells. The periphery of the germinal centre is outlined by several brilliantly autofluorescent granules. Stained by a single layer method with FITC-labelled sheep anti-HSA.

x 2000

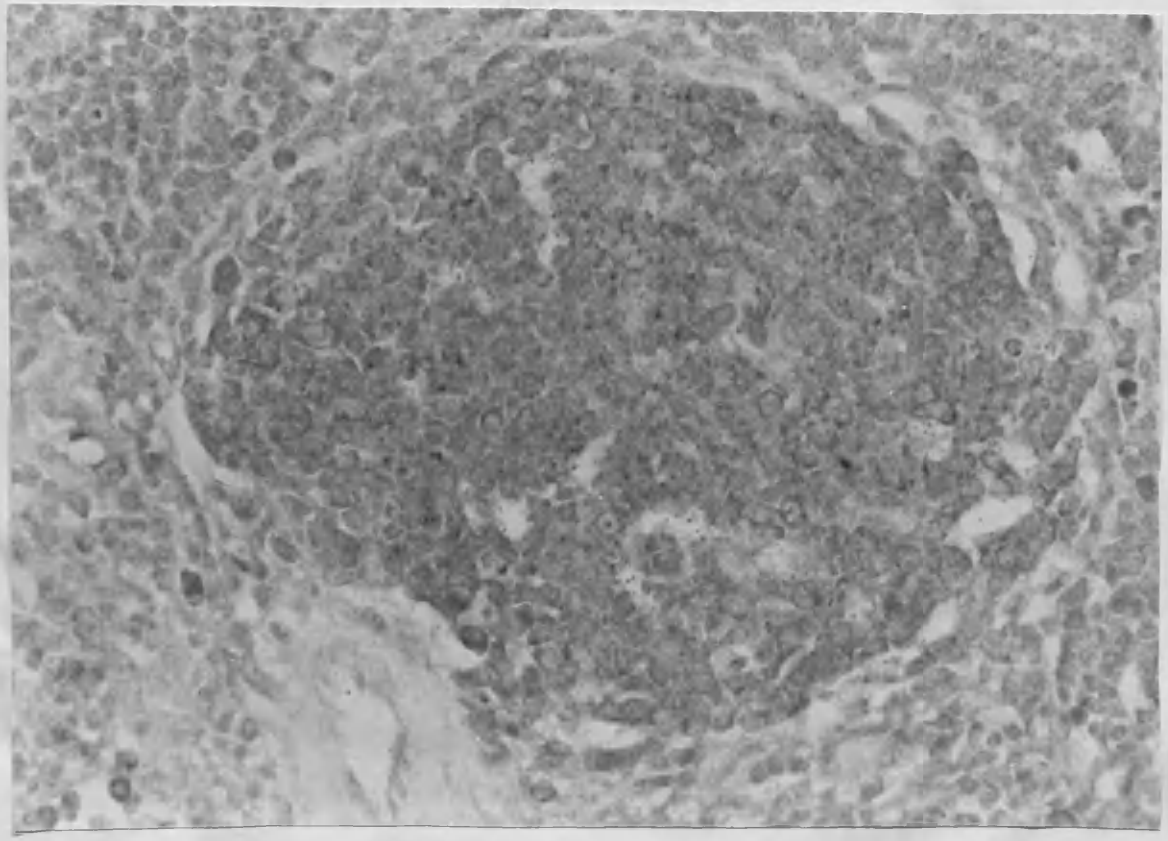


Fig. 6.1.1.

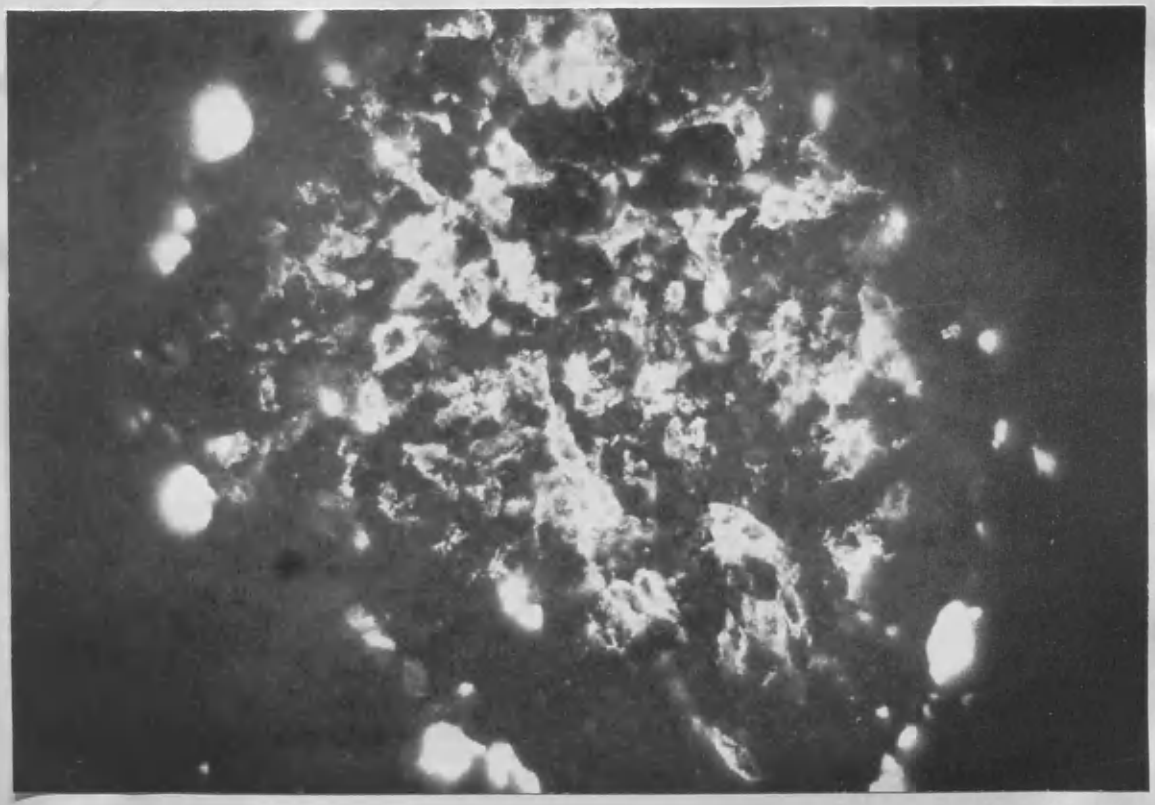


Fig. 6.1.2.

detail (White, 1963; White, French and Stark, 1970). Factors which aid or interfere with the normal formation of germinal centres in the chicken spleen have been assessed in a subsequent paper (White et al., 1975). Briefly these events are:

1) an intravenously injected protein antigen such as human serum albumin (HSA) circulates in the blood until antibody is synthesized and the resulting antigen-antibody complexes then gain access (at about 48 hours after injection) to the white pulp of the spleen. The complexes can be seen on the bodies and cytoplasmic dendritic extensions of certain cells in the peri-ellipsoidal cuff, which have been called "dendritic cells" (White, 1963).

2) the antigen-bearing dendritic cells migrate toward the point of division of the central arteriole into the penicillary arterioles. This is the site of origin of the germinal centres which appear as small balls of cells from 72 hours after injection. These small cellular aggregates appear to grow by the progressive inclusion of antigen-bearing dendritic cells and B-lymphocytes. Antigen-bearing cells subsequently appear in sections at day 6 after injection wholly within the germinal centres and may be detected in these centres for at least 28 days (personal observations). The evidence that the germinal centre involves specific B cells, which associate with the dendritic cells, is as follows:

1) non-antigenic particles, although taken up by the macrophages of the spleen, are not included in the germinal centre

2) birds bursectomised at hatch and subsequently X-irradiated fail to develop germinal centres even after antigen injection

3) in vitro labelled autologous (De Sousa et al., 1973) or syngeneic (Durkin et al., 1971) bursa cells injected intravenously into recipients become included in typical germinal centres within 24 hours

4) a proportion of germinal centres formed after injection of antigen are found to contain a high proportion of specific antibody-containing cells from 13 days onward (White et al., 1970).

Thus the typical germinal centre consists of antigen-bearing dendritic cells, interspersed with, and in close contact to, B lymphocytes, and through their dendrites, to each other. This is confirmed by the appearance of germinal centres in electron micrographs which indicate very close membrane contact between component B-lymphocytes and dendritic cells, possibly maintained by the presence of antigen as a complex with antibody (See Wallace, 1976). The B cells within the centres are packed with ribosomes but contain no endoplasmic reticulum, i.e. they are not differentiating to plasma cells and may be in a state of tolerance under the influence of the antigen-antibody complexes.

6.1.3. Distribution

Most studies have been carried out on the germinal centres of the secondary (peripheral) lymphoid tissues such as the spleen or

Fig. 6.1.3.

Photomicrograph of two germinal centres in a thin section of the caecal tonsil. The centres share the same morphological and staining characteristics as those in the spleen.

U.P. x 2000

Fig. 6.1.4.

Photomicrograph of germinal centre in section of chicken liver. The germinal centre forms part of a lymphoid follicle which is clearly distinct from the normal hepatic tissue.

U.P. x 2000

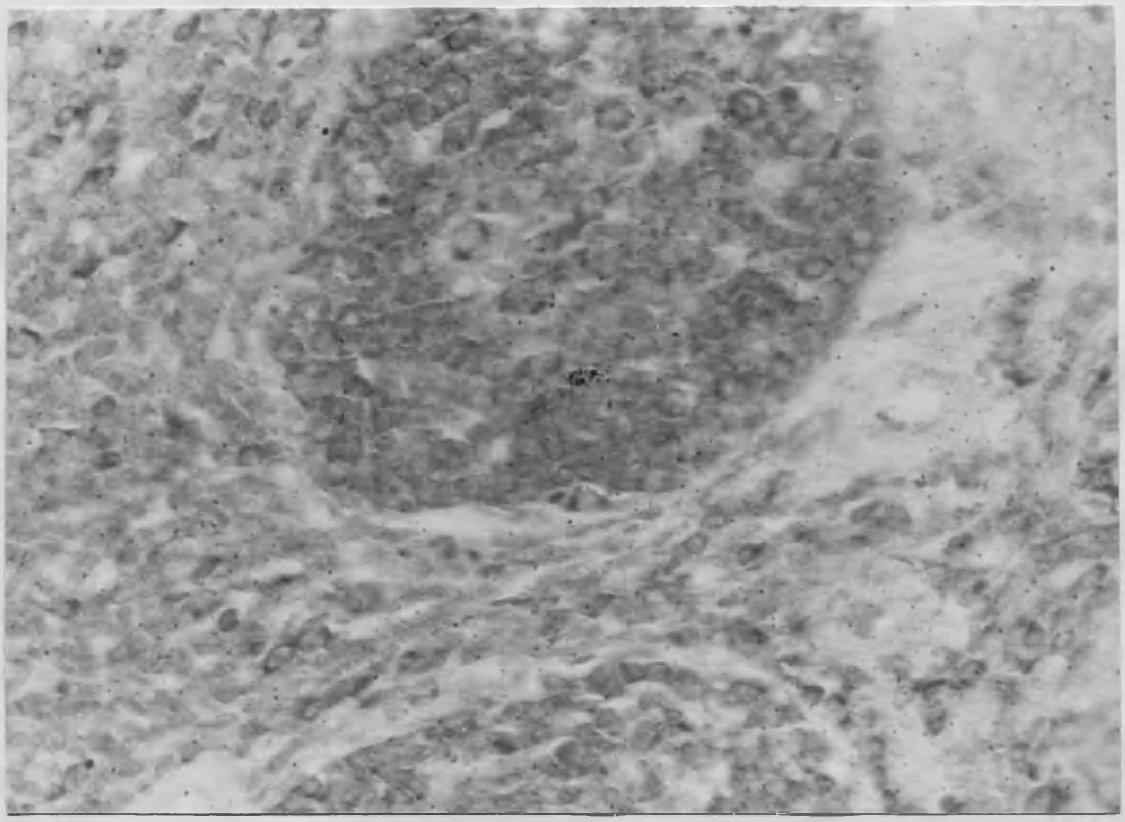


Fig. 6.1.3.

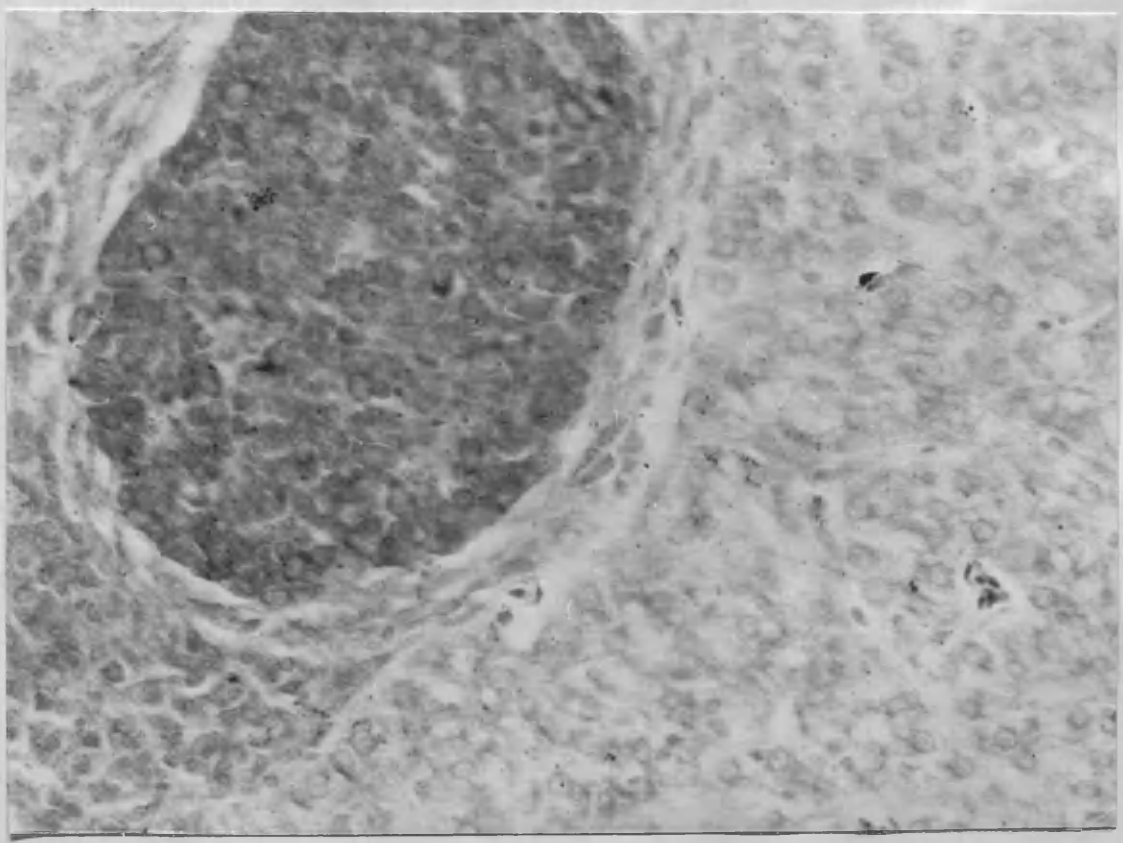


Fig. 6.1.4.

gut associated lymphoid tissue, especially the caecal tonsils (Fig. 6.1.3.). However lymphoid areas, and associated germinal centres have also been described in a wide range of non-lymphoid tissues and organs of the chicken such as the thyroid, pituitary, gonads and adrenal (Wheeler, 1950); the liver (Fig.6.1.4.) and kidney (Lucas et al., 1954); the pancreas (Fig.6.1.5.) (Lucas, 1949, Lucas and Oakberg, 1950) and nerves (Oakberg, 1950).

In addition it is now known (Isakovic and Jankovic, 1967) that germinal centres normally occur in one of the primary (central) lymphoid organs of the bird, namely the thymus (Fig.6.1.6.).

Mural Lymphoid Nodules

The members of the species, Gallus domesticus do not possess lymph nodes, and in this they differ from ducks and other aquatic species, which have two rudimentary pairs of nodes, the cervico thoracic nodes at the thoracic inlet, and the lumbar nodes in the abdomen on either side of the abdominal aorta. However Kiharo and Maito (1933) showed that, as well as lymph nodes, ducks possess lymphoid nodules scattered irregularly along the main lymphatic vessels of the neck, wing and leg. Kondo (1937a) subsequently turned to the domestic fowl in which every lymph vessel examined had similar nodules. In a further study (Kondo, 1937b) he examined thirteen avian species and found lymphoid nodules associated with the lymphatic vessels of them all.

Biggs (1957) has characterised the size, number, and distribution of these lymphoid nodules in the chicken. They vary in size from

Fig. 6.1.5.

Photomicrograph of chicken pancreas section showing two germinal centres in an area of lymphoid infiltration, surrounded by pancreatic a cini.

U.P. x 1250

Fig. 6.1.6.

Two germinal centres in thymic lobule of an adult chicken. The characteristic features of these structures enable identification even in primary lymphoid tissues such as the thymus.

U.P. x 1250

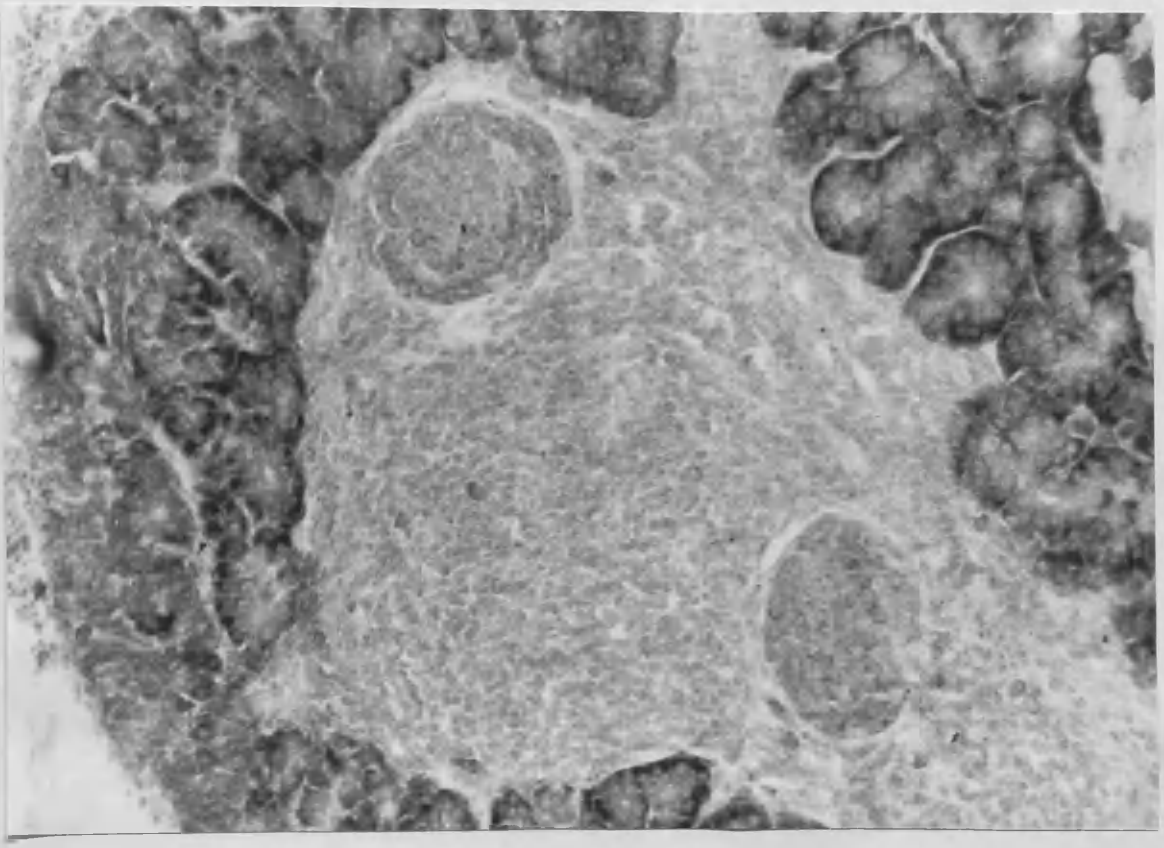


Fig. 6.1.5.

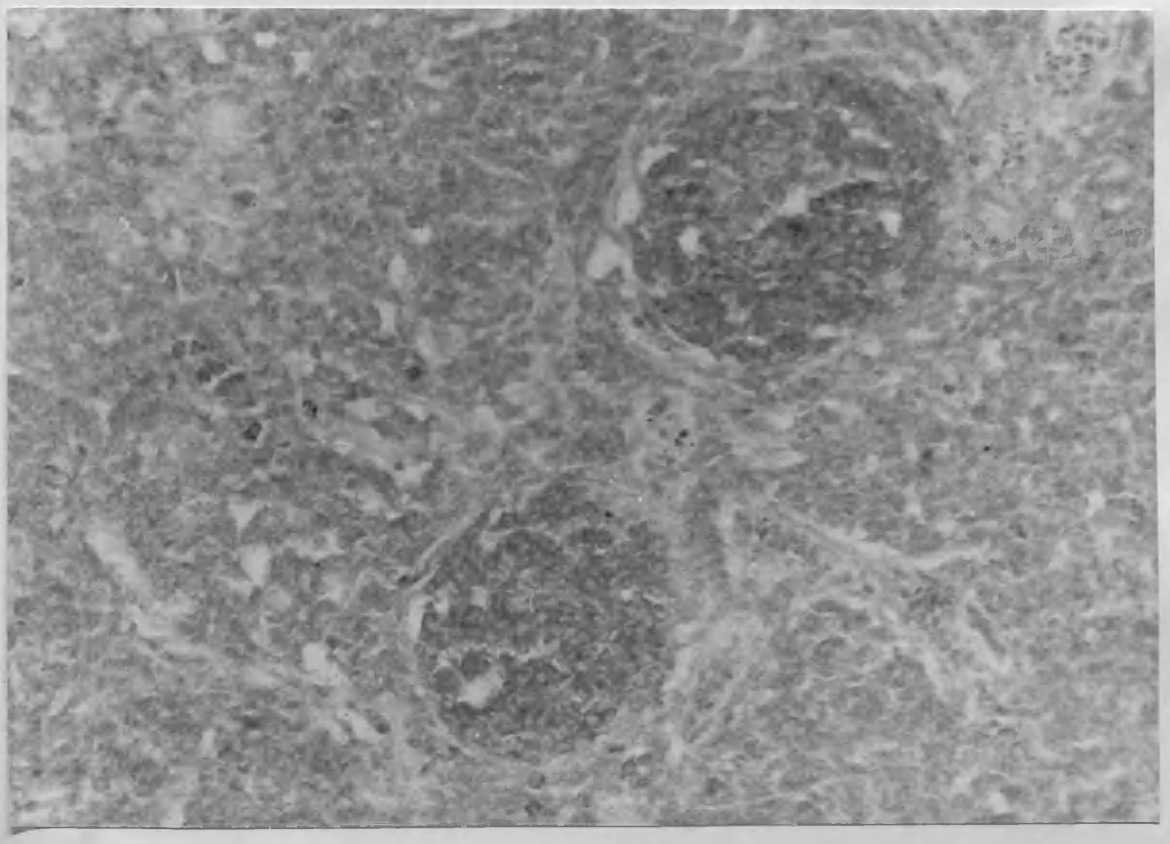


Fig. 6.1.6.

0.1 to 2.5 mm in length, and occur at a rate of 1.25 per cm length of lymph vessel. The nodules may occasionally be seen as pale brown bodies attached firmly to the lymphatic vessel and ususally embedded in fatty tissue. Stained transverse sections show that the nodules are usually confined to the wall of the vessel and may protrude into the lumen, and this has led Biggs (1957) to propose the name mural lymphoid nodules. The bulk of the nodule is composed of sheets of small lymphocytes but germinal centres or "secondary nodules" can be seen.

That the mural lymphoid nodules are a primitive equivalent of the mammalian lymph node may be inferred from the study of the Australian echidna, Tachyglossus aculeatus, a close relative of the extinct ancestors of higher mammals (Diener et al., 1967). This animal does not possess lymph nodes but a structure, the lymphnodule, intermediate between the chicken mural lymphoid nodule and mammalian lymph node. These structures were mainly associated with the blood vessels of the thoracic, cervical and pelvic regions and mesentery of the gut. The nodules, each of a diameter of approximately 0.2 - 2mm form large clusters within a lymphatic plexus. The suggestion has been made by the authors that the transition from the lymph nodule system to the lymph node system resulted in greater efficiency in the immune response.

6.2. Antigen localisation to germinal centres

It has been shown in the preceding section that germinal centres may occur as part of lymphoid accumulations in many tissues of the chicken, either along the lymphatics or in lymphoid areas of the tissues. The localisation of antigen to germinal centres in the chicken spleen has been extensively investigated by White and his colleagues (White, 1963; White et al., 1970; White et al., 1975), and the results have been outlined in the introduction. It was felt however that a repeat of these studies might lead to additional information, when considered from the view point of local germinal centre formation. Therefore groups of birds were given both HGG and I^{125}-labelled HSA in varying doses intravenously and the localisation of the antigen followed by autoradiography and immunofluorescence; at 3 days, 5 days and 7 days after injection.

A gross impression of antigen localisation in the spleen at 3 days may be obtained from the dark-ground autoradiograph in Fig.6.2.1. The piece of tissue is a whole spleen section and the numerous small white circles correspond to the localisation of HSA at the ellipsoids

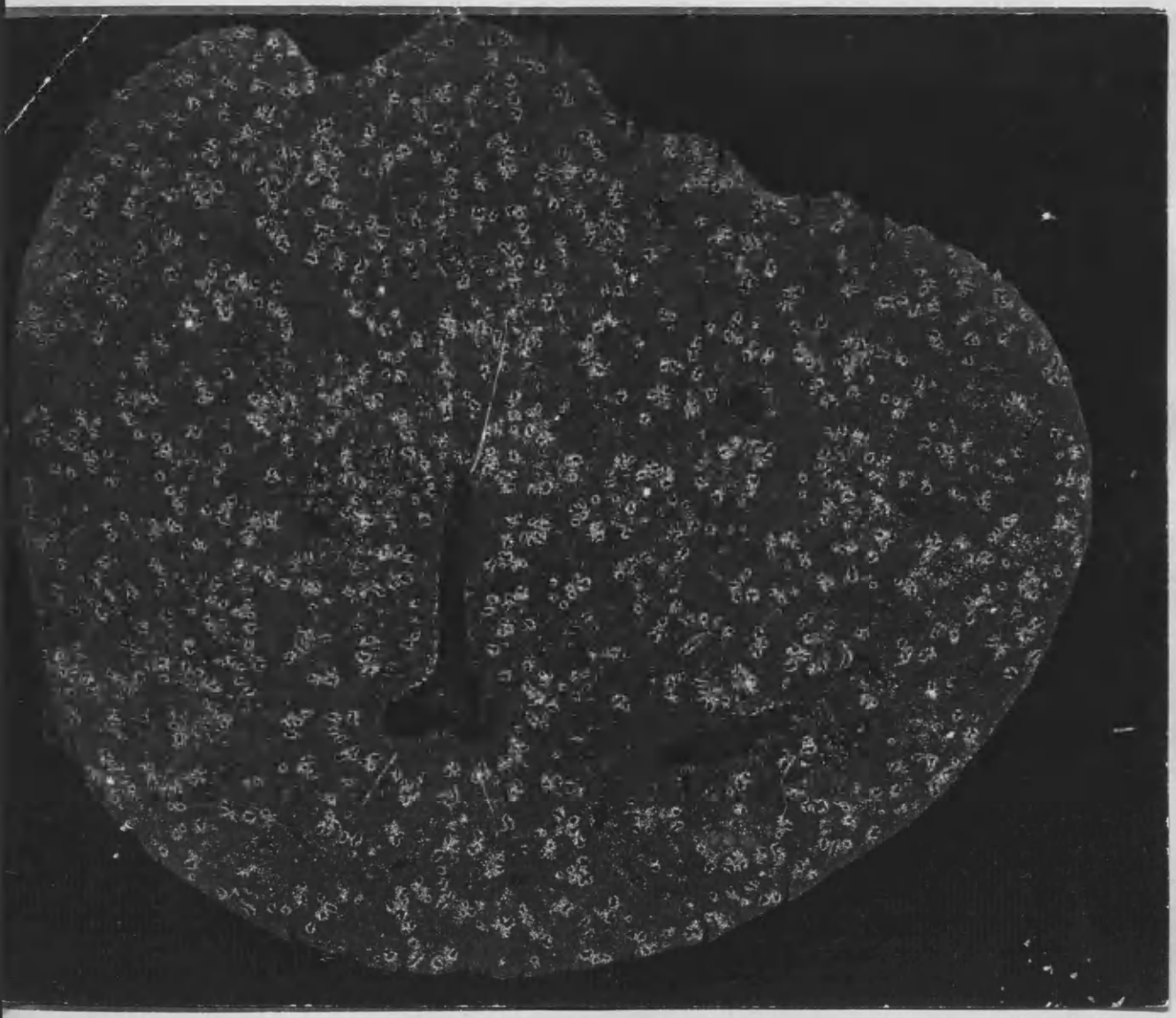


Fig. 6.2.1.

Autoradiograph of chicken spleen section 3 days after i.v. injection of I^{125}-labelled HSA. The dark-field photomicrograph shows localisation of antigen to the periphery of the Schweigger-Seidal sheaths (seen here as small white rings) which occur at the boundary between the red and white pulp.

Dark-field x 15

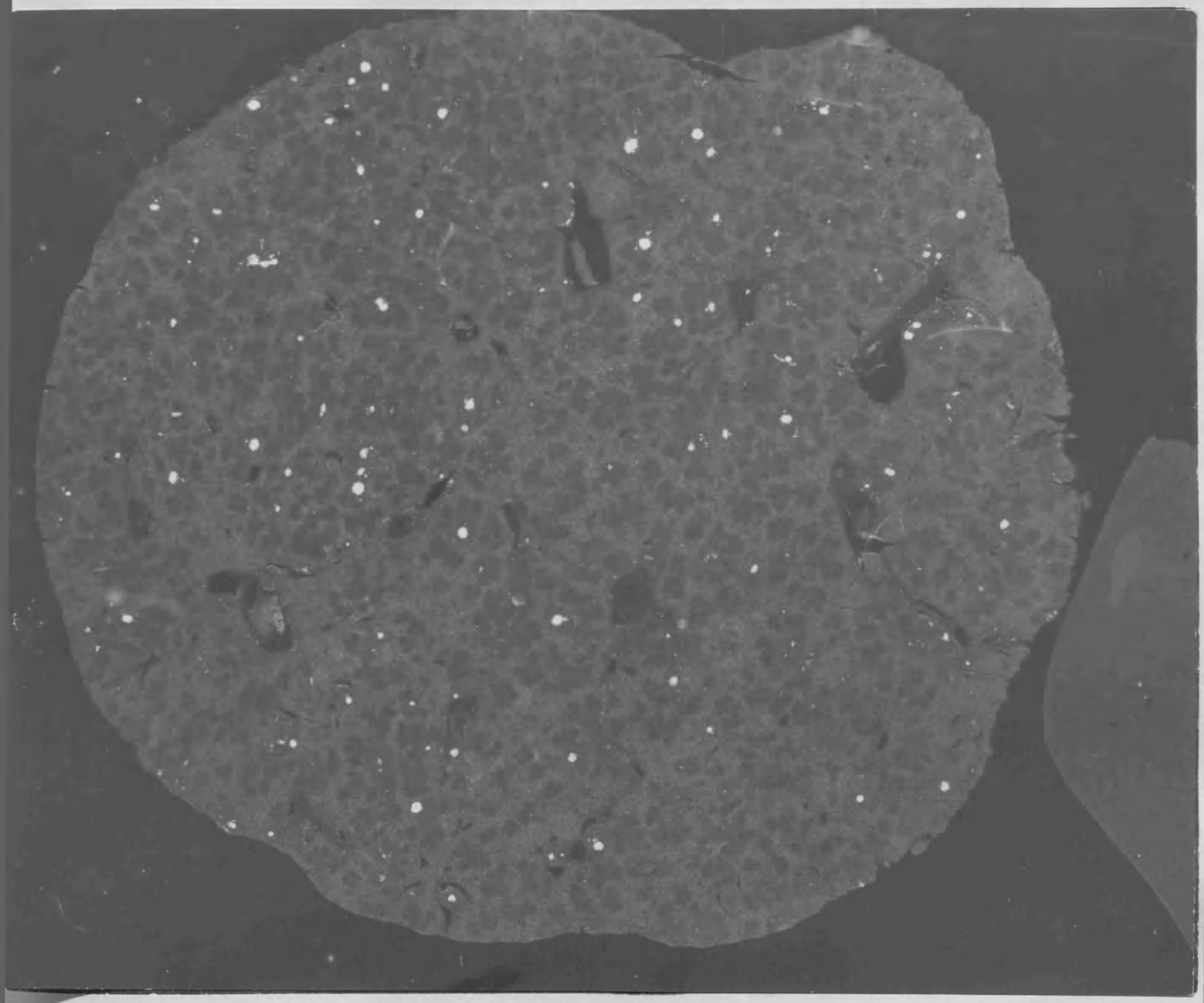


Fig. 6.2.2.

Autoradiograph of chicken spleen 5 days after i.v. injection of I^{125}-labelled HSA. The dark field micrograph shows localisation of the antigen within germinal centres (seen here as small white stars) in the white pulp areas. Note that little or no antigen can be demonstrated in the region of the Schweigger-Seidel sheaths at this time.

Dark field x 15

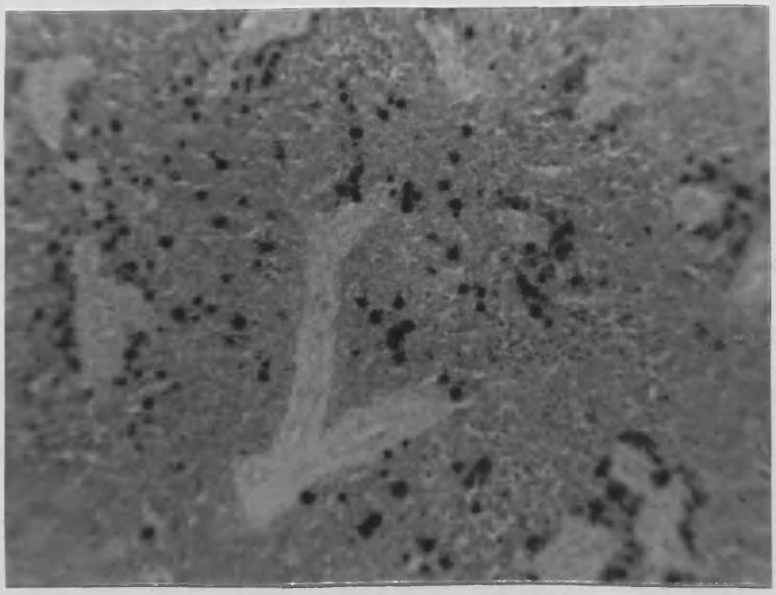


Fig. 6.2.3.

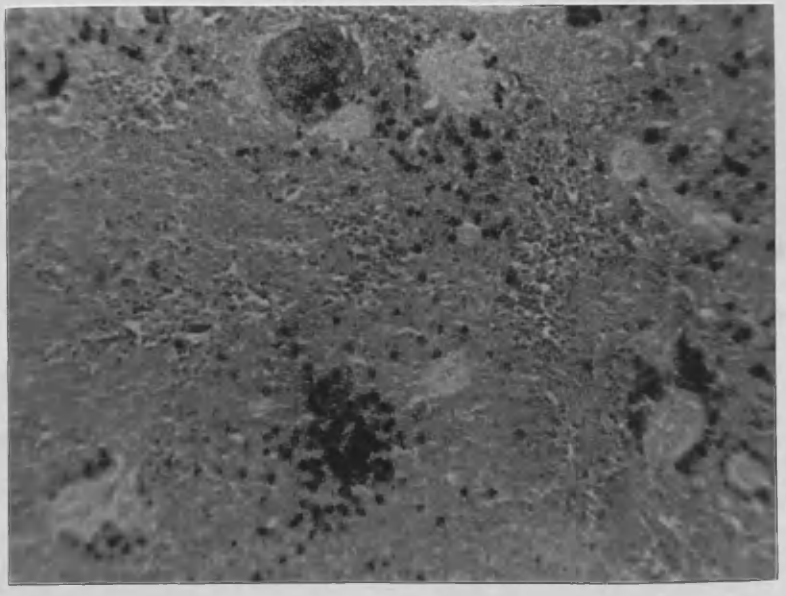


Fig. 6.2.4.

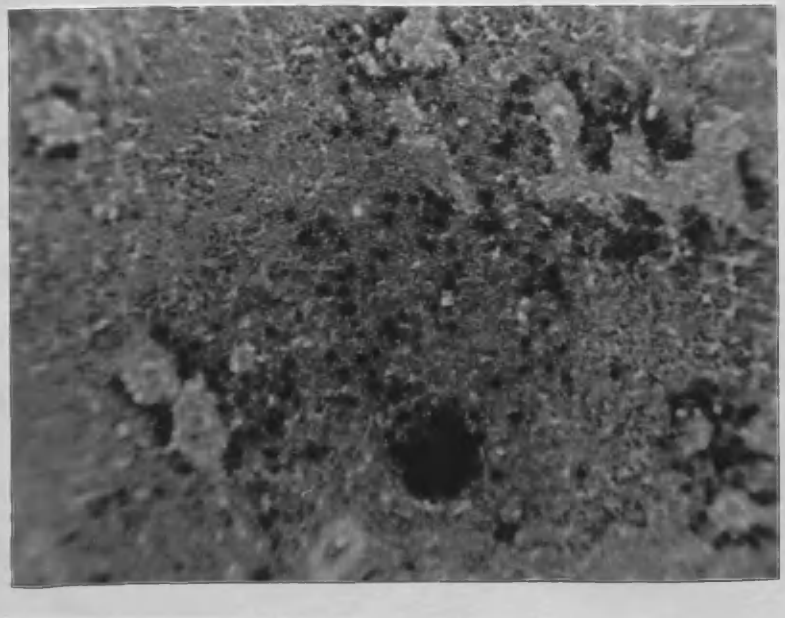


Fig. 6.2.5.

(Schweigger-Seidal sheaths)in the form of antigen-antibody complexes. Fig. 6.2.2. demonstrates the appearance of a similar spleen section at 5 days. The ellipsoidal pattern has disappeared entirely and the small bright white dots correspond to antigen-containing germinal centres. There has been a massive loss of antigen between three and five days with only a small fraction left in the germinal centres. If one examines these spleen sections at a higher magnification the following points may be noted. The ellipsoidal pattern seen at 3 days corresponds to numerous macrophage-like cells clustered round the ellipsoids, bearing labelled antigen on their surfaces or in the cytoplasm (Fig.6.2.3/4/5). The cells with antigen on their surface correspond to those described by White as dendritic cells. Several of these cells can be seen to be strung out between the ellipsoids and white pulp islands along the course of penicillary vessels. Few germinal centres are visible at this time though a number of dendritic cells have clustered at the fork of a penicillary and central arteriole, a common site for germinal centres. In Fig. 6.2.6 . one of the white dots in Fig. 6.2.2. has been magnified to reveal the characteristic antigen localisation in a germinal centre. Several dendritic cells, shown by overlying black grain foci, have come together to form a germinal centre. Fig. 6.2.7. shows a similar centre resulting from a smaller dose of antigen (1 mgm) in which greater cellular detail is evident with less overlying grain numbers. The dendritic appearance of each antigen-bearing cell can be clearly seen. This corresponds well with the immunofluorescent picture of a similar germinal centre stained with sheep anti-HSA-FITC 7 days after injection of

Fig. 6.2.6.

Autoradiograph of chicken spleen 5 days after i.v. injection of 10 mgm HSA-I^{125}. Numerous heavily labelled dendritic cells (seen here as black clumps) have agglutinated to form a germinal centre tangential to a penicillary arteriole. Antigen in the wall of the vessel may be in the form of deposits or on cells migrating from the circulation.

U.P. x 2000

Fig. 6.2.7.

Autoradiograph of chicken spleen 7 days after injection of 1 mgm HGG-I^{125} i.v. In this case there are far fewer silver grains and greater cellular detail is visible. The surface pattern of the labelled antigen on dendritic cells can be clearly seen.

U.P. x 2000

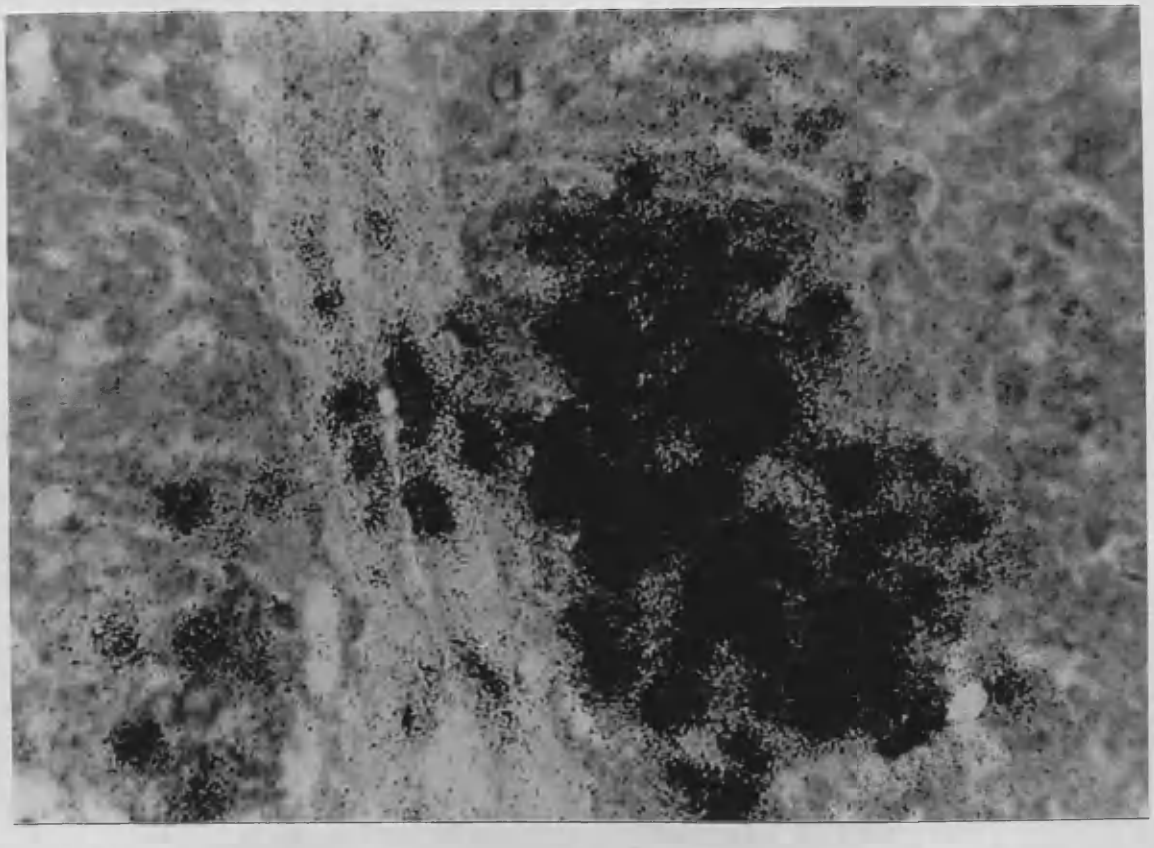


Fig. 6.2.6.

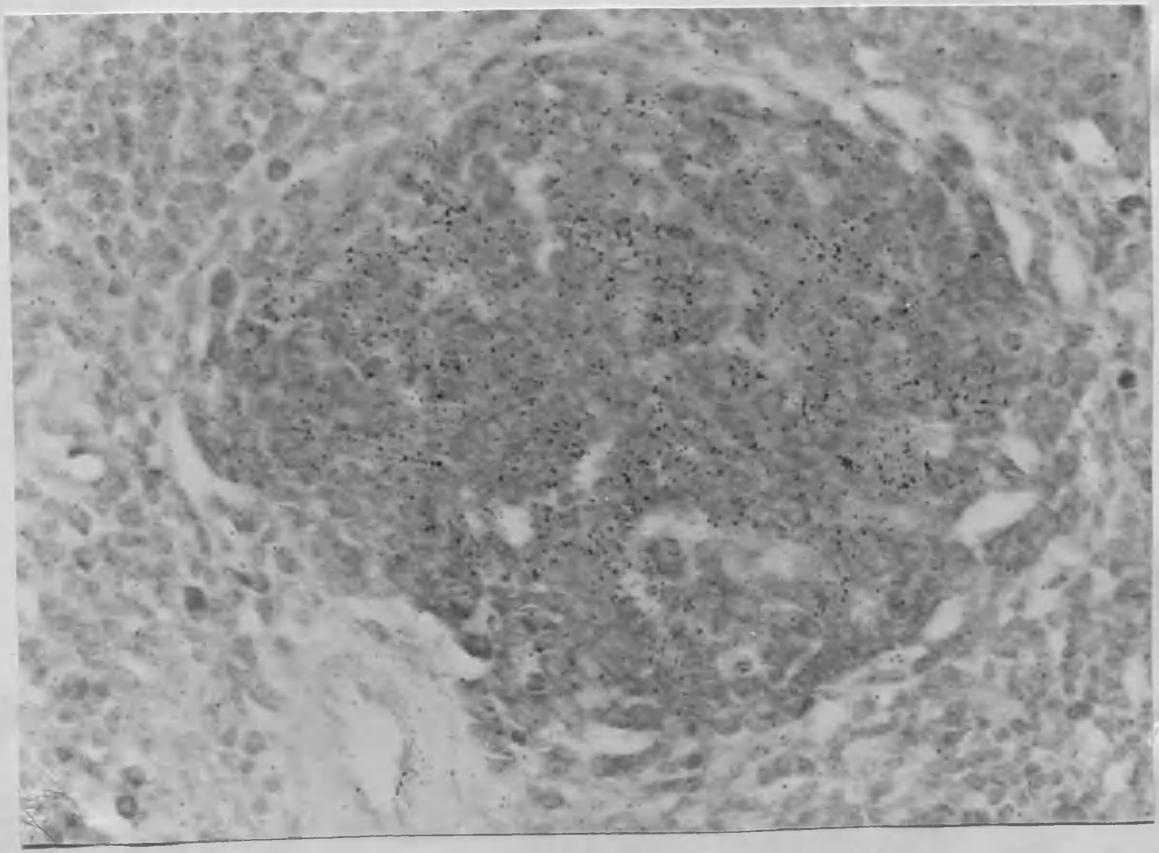


Fig. 6.2.7.

Fig. 6.2.8.

Autoradiograph of chicken spleen 5 days after i.v. administration of HSA-I^{125}. A newly-formed HSA-containing germinal centre is seen to the left of a pre-formed centre with little or no labelled antigen.

U.P. x 200

Fig. 6.2.9.

As above. Two germinal centres are seen in close proximity. The upper centre is newly-formed and is entirely comprised of HSA-bearing dendritic cells while the lower, pre-formed structure has included only a few labelled cells.

U.P. x 200

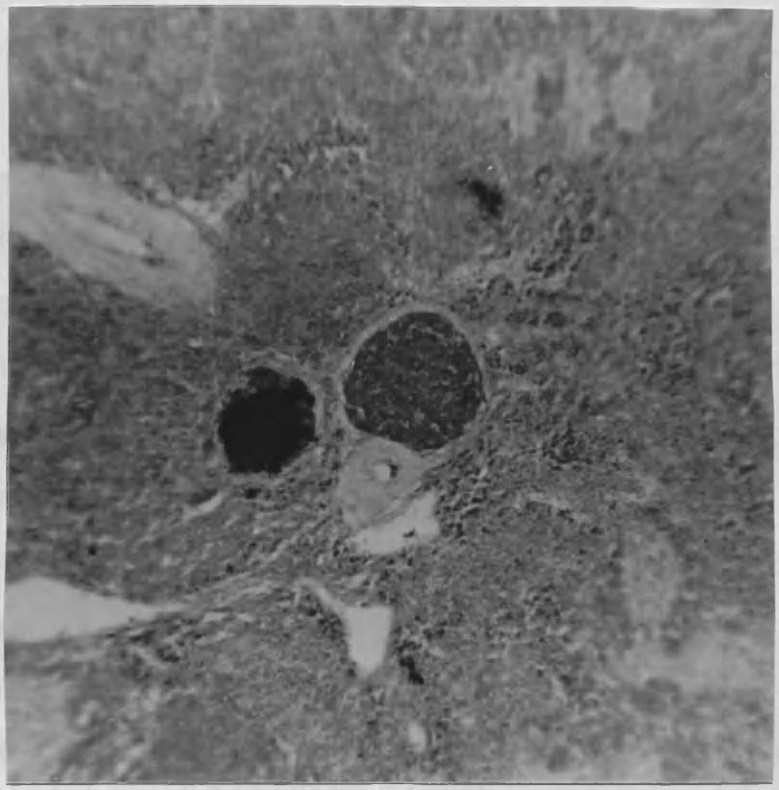


Fig. 6.2.8.

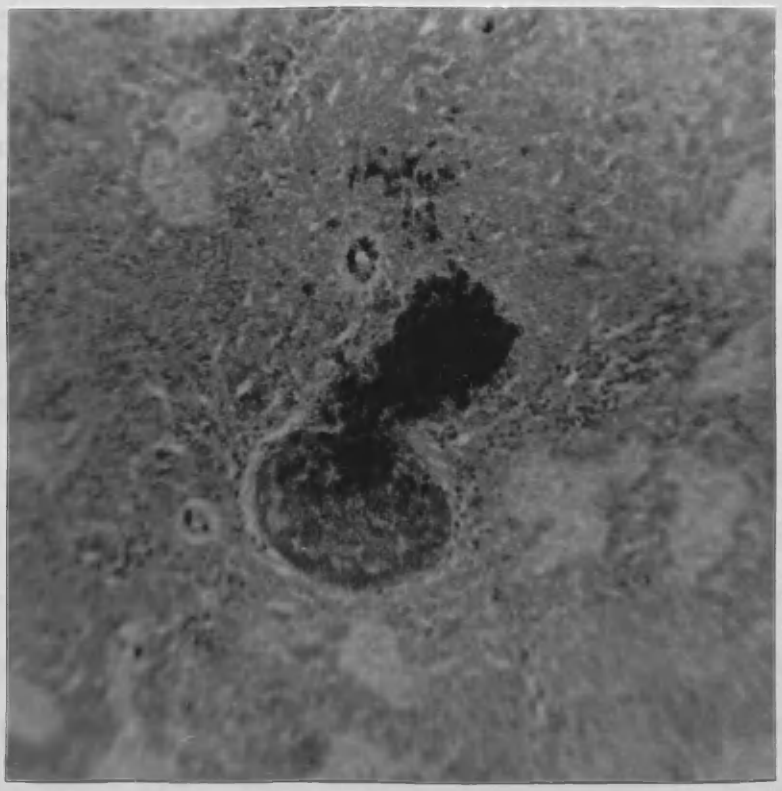


Fig. 6.2.9.

10 mgms HSA (Fig. 6.1.2.). The antigen is seen on the surface and following the outline of the dendritic cells.

One aspect of the antigen localisation which has not often been stressed is the appearance, for long periods after administration, of small deposits of antigen on the wall of arterioles closest to an adjacent germinal centre (Fig. 6.2.6/7). These deposits persist throughout the life-time of the local germinal centre. Their role is unclear, perhaps they function to direct the traffic of specific antigen reactive cells to the adjacent centre. Another possibility is that these are not deposits but antigen-bearing cells passing through the endothelium, short circuiting the normal route via the ellipsoids and white pulp, and passing directly into the germinal centre.

The difference between pre-formed and newly formed germinal centres is shown in Fig. 6.2.8. Here germinal centres which have presumably formed prior to the HSA inoculation are seen adjacent to newly formed HSA-containing centres. The preformed centres have little HSA, the new centres are almost entirely occupied by HSA-bearing dendritic cells.

6.3. Double antigen localisation to germinal centres

Considerable interest centres round the question of whether germinal centres are specific for particular antigens. The evidence that germinal centres in the chicken spleen involve specific cells to antigen on the dendritic cells is that a proportion of germinal centres formed after injection are found to contain a high proportion of specific antibody containing cells. (See Fig.1.2.s.part I). This does not exclude the possibility however, that more than one antigen

could gain access to a germinal centre, especially as the antigen-dendritic cell connection is mediated through the Fc portion of antibody, i.e. it is a non-specific process with regard to the antigen. It was of interest therefore to know if more than one antigen would localise to one germinal centre or if different antigens would be found in different centres.

Consequently the following experiment was carried out. Eight adult chickens, of both sexes, were used. Two birds received a single intravenous injection of 1 mg of HGG monomer, and two birds a single intravenous injection of 1 mg I^{125}-labelled HSA. The remaining four birds each received two injections: the first of 1 mg of HGG monomer followed immediately by the second of 1 mg I^{125}-labelled HSA. Birds were killed either at 3 or 6 days after immunisation. The presence of the two antigens was revealed on the same cryostat tissue sections by the technique of combined immunofluorescence and autoradiography.

a) Normal Immunofluorescence

Cryostat sections of the spleens of birds killed three days after the injection of either I^{125}-labelled HSA or monomeric HGG showed the localisation of these antigens by direct immunofluorescence (using either FITC-labelled anti-HSA or FITC-labelled anti-HGG) upon the surface of dendritic cells within the white pulp areas. Such antigen-bearing cells were found as free individuals scattered throughout the white pulp and also in early germinal centres in the angle of bifurcation of the penicillary from the central arteriole. At 5 days after injection almost all antigen-bearing dendritic cells were found within the circular confines of germinal centres (Fig. 6.3.1.)

Fig. 6.3.1.

Fluorescence micrograph of a germinal centre in the white pulp of the spleen of a bird killed 6 days after the simultaneous intravenous injection of I^{125}-labelled HSA and HGG monomer in saline. The distribution of HGG monomer upon the surface of dendritic cells is shown by the apple-green fluorescence. The outline of the centre is indicated by the surrounding autofluorescent granules which appear as dense white oval bodies and are characteristically found in this region.

x 720

Fig. 6.3.2.

Dark ground autoradiograph of the same germinal centre shown in Fig. 6.3.1. The distribution of I^{125}-labelled HSA upon the surface of dendritic cells is shown by the overlying silver grains.

x 720

Fig. 6.3.3.

Combined autoradiograph and fluorescence micrograph of the same germinal centre shown in Figs. 6.3.1. and 2. The distribution of fluorescently labelled HGG monomer and I^{125}-labelled HSA suggests that these two antigens are present on the same dendritic cells.

x 720

Fig. 6.3.1.

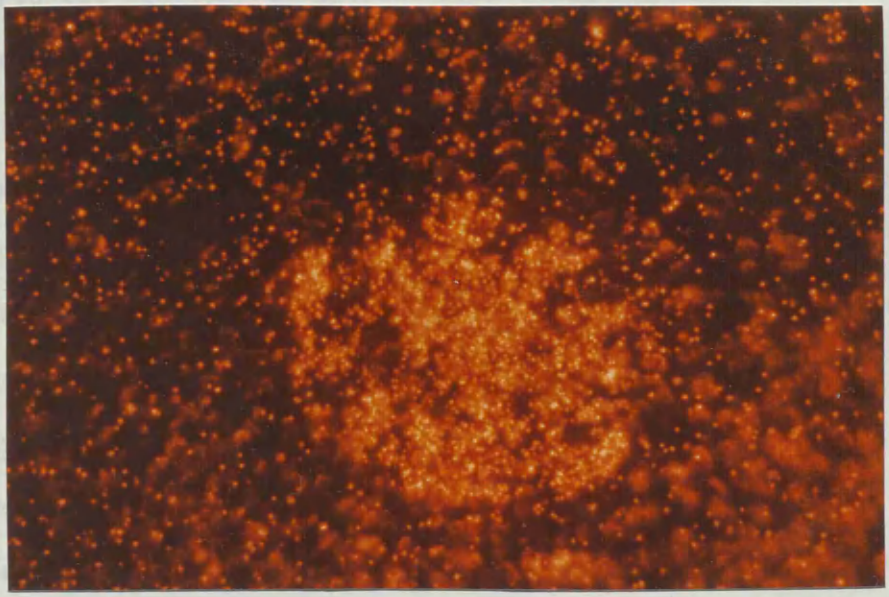


Fig. 6.3.2.

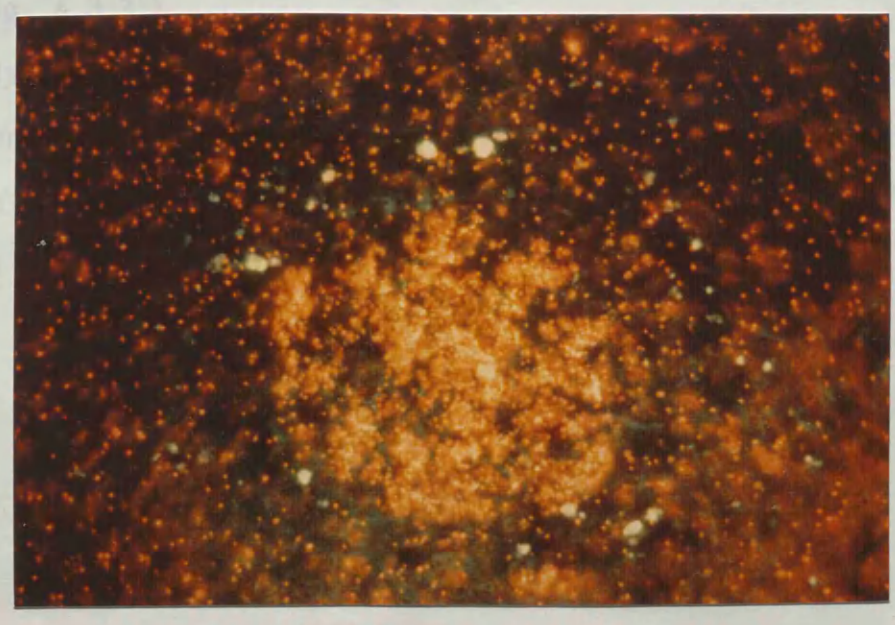


Fig. 6.3.3.

b) Normal autoradiography

The histological localisation of I^{125}-labelled HSA by autoradiography of cryostat sections of the spleen was identical to that seen by immunofluorescence (Fig.6.3.2.). The black silver grains appear here as red due to the use of a red filter and dark ground illumination.

c) Combined immunofluorescence and autoradiography

Cryostat sections of spleens from birds injected with both I^{125}-labelled HSA and monomeric HGG stained first with fluorescein-labelled anti-HGG antibody and then subjected to autoradiography revealed the presence of dendritic cells carrying both antigens as well as dendritic cells positive for only one antigen within the white pulp at three days after injection. The majority of germinal centre clusters contained doubly-labelled dendritic cells. One germinal centre group containing only HGG-bearing dendritic cells was found. At five days after injection germinal centres contained dendritic cells bearing both I^{125}-labelled HSA and monomeric HGG, so that, in general, the overlying silver grains followed the pattern of fluorescence (Fig. 6.3.3.).

Finally the cryostat sections of spleens of birds injected with only monomeric HGG processed as above did not contain any cells with overlying silver grains and vice versa, sections from birds injected with only I^{125}-labelled HSA did not contain any fluorescing cells.

6.4. Homing of ^{3}H-adenosine-labelled autologous bursa cells to germinal centres in the chicken spleen.

As mentioned earlier in the introduction to germinal centre function (page 142) homing has been demonstrated of both syngeneic (Durkin et al, 1971) and autologous (De Sousa et al, 1973) labelled bursa cells to germinal centres of the chicken spleen. This was in contrast to the previous results of Linna and coworkers (1969) who failed to show such homing with in situ labelled bursa cells.

These observations are of considerable interest in regard to the role of local germinal centre formation in adjuvant granulomata, especially in view of the fact that germinal centre cells occasionally transform into specific antibody-containing plasmablast-like cells (White et al, 1970). These studies were therefore repeated and the pattern of bursa cell homing contrasted with that of autologous thymus-derived cells.

Two groups of young (six week old) chickens were subjected to either partial thymectomy or partial bursectomy and an inoculum of 5×10^{7}, ^{3}H-adenosine-labelled cells prepared from each bird and re-injected intravenously (each bird receiving its own cells). The birds were killed exactly 24 hours later and the spleens processed for autoradiography.

The results were extremely clear cut. Approximately 50% of the labelled bursa cells counted on spleen sections were found in germinal centres in the white pulp and indeed formed the major proportion of the cells seen in these structures (Figs. 6.4.1. and 6.4.2.). Densely labelled bursacytes were scattered through the central region

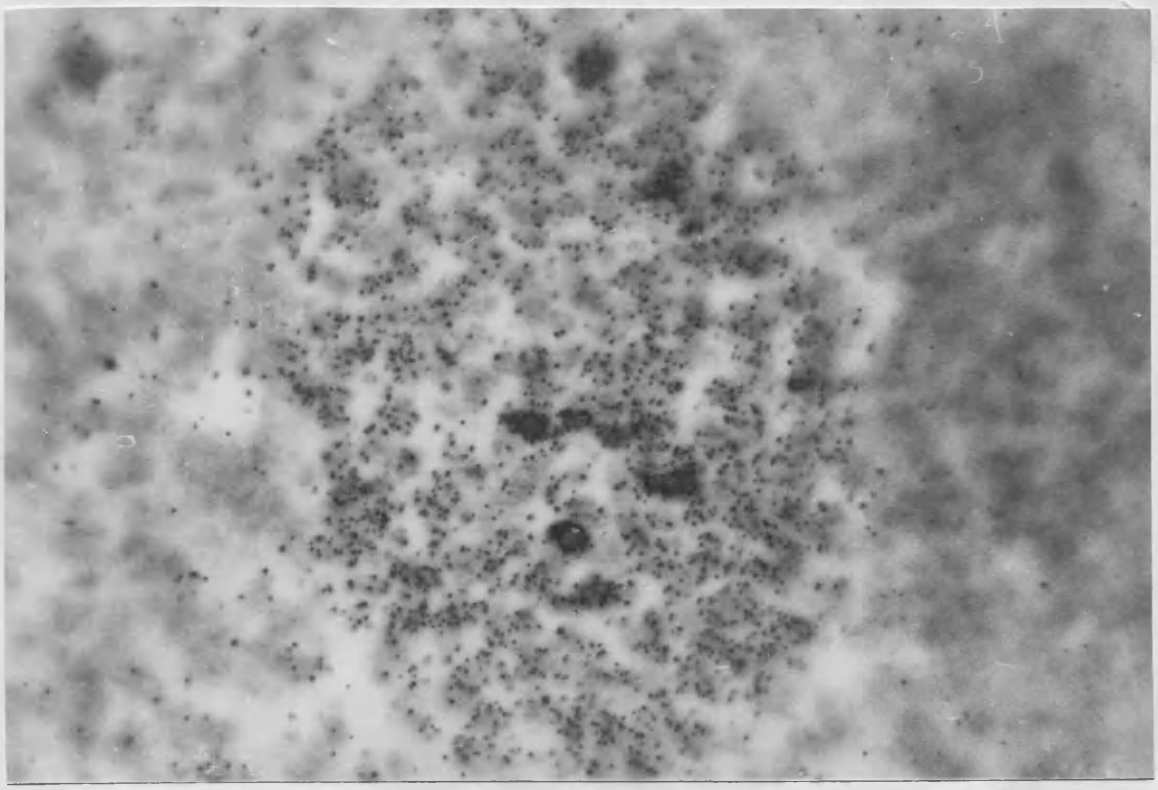


Fig. 6.4.1. Autoradiograph of a germinal centre in chicken spleen 24 hours after the i.v. transfusion of 5×10^7 autologous, ^{3}H-adenosine labelled bursa cells. Note the presence of heavily labelled cells at the centre and more lightly labelled cells toward the periphery, suggesting that cells had divided after arrival in the germinal centre site.

x 1200 U.P.

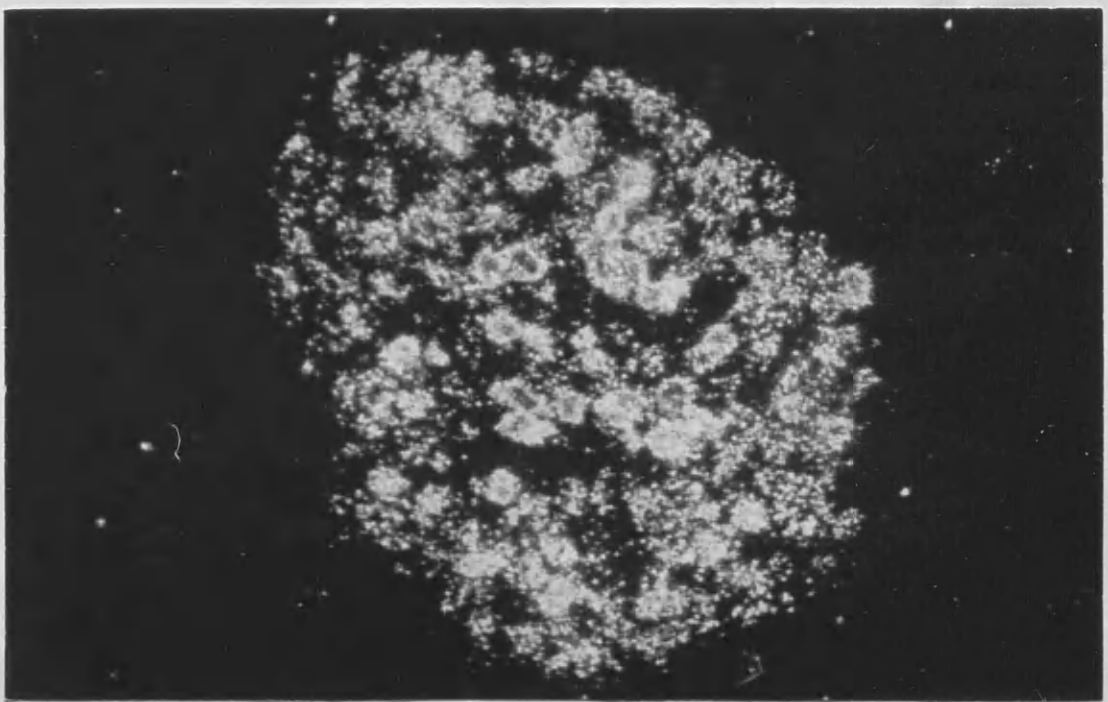


Fig. 6.4.2. Dark-field autoradiograph of similar centre. x 1200.

of the germinal centre with less heavily labelled cells toward the periphery. The lightly labelled cells presumably represent bursa cells which have divided in situ, perhaps in response to the antigens on the surface of nearby dendritic cells. The edges of the germinal centres were easily distinguished by the sharp demarcation of silver grains from the surrounding tissue. Other labelled bursa cells were seen (in roughly equal proportions) surrounding the ellipsoids, in the red pulp, and in the white pulp.

In contrast few, if any, labelled thymus cells were seen in germinal centres. Instead these cells tended to localise mainly to the peri-arteriolar sheaths and to the white pulp areas surrounding germinal centres. Occasional cells (Fig. 6.4.3.) could be seen at the boundary separating the germinal centres from the remaining white pulp.

These results accord well with those of Durkin and her colleagues who found a similar number of bursa cells (50%) homing to the splenic germinal centres of normal,i.e. unimmunised,chickens.

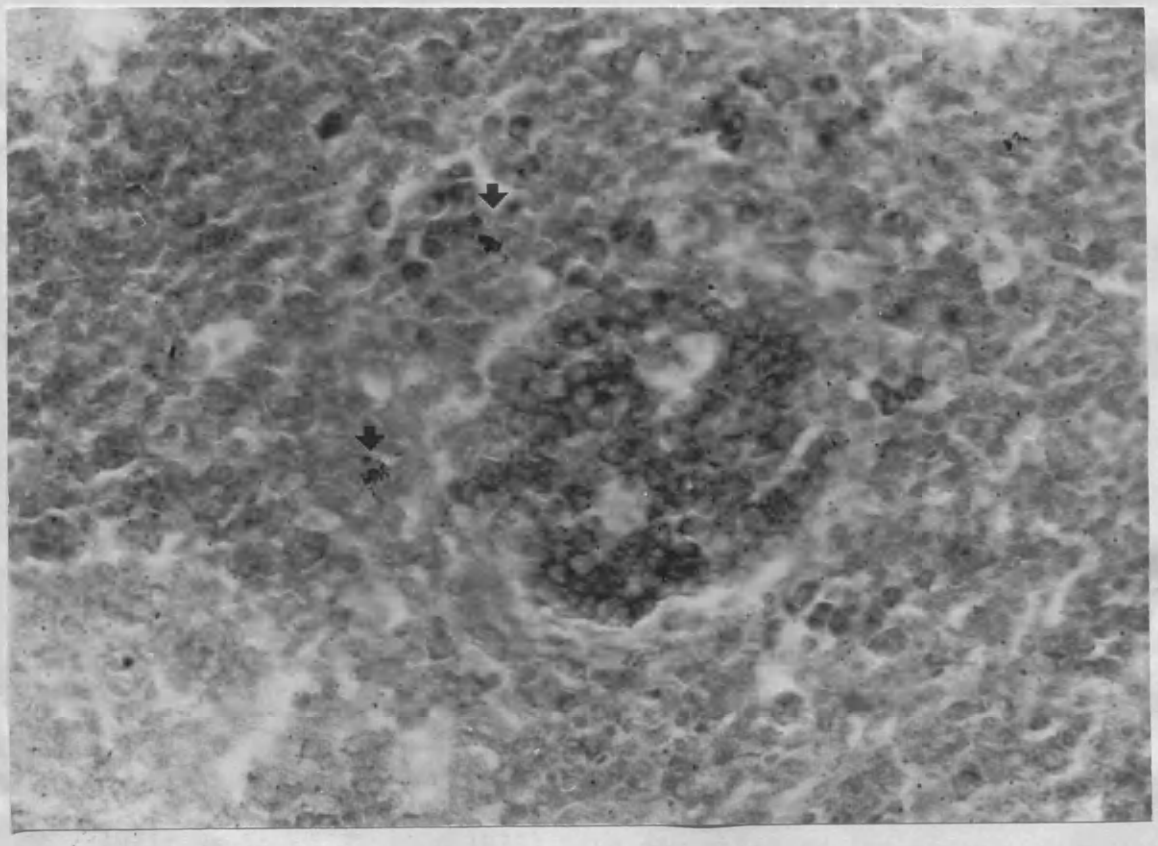


Fig. 6.4.3. Autoradiograph of a germinal centre in a chicken spleen 24 hours after the i.v. transfusion of 5×10^7, ^{3}H-adenosine-labelled thymus cells. No labelled cells are visible within the centre though two may be distinguished just beyond the germinal centre boundary on the left (arrowed).

x 1200 U.P.

6.5. Homing of bursa cells to HSA-containing germinal centres in the chicken spleen

The localisation of a soluble protein antigen, human serum albumin (HSA) in the chicken spleen has been extensively characterised by White and his colleagues (White, 1963; White et al., 1970; White et al., 1975) and has been discussed in a previous section (6.2.). Between 88 hours and 6 days after intravenous injection antigen on the surface of dendritic cells was seen within germinal centres in the white pulp of the spleen, and persisted in these sites for several weeks. This led to the hypothesis that germinal centres are formed *de novo* following the progressive capture of immunologically competent lymphocytes by antigen-bearing dendritic cells.

Studies on the traffic of bursa cells in the chicken (Durkin et al., 1971; De Sousa et al., 1973) have demonstrated the homing of both *in vivo* and *in vitro* labelled bursa cells to germinal centres in the chicken spleen. (See also Section 6.4.). A high proportion of *in vitro* labelled bursa cells (50% of those counted - spleen sections) are found in germinal centres 24 hours after intravenous injection. This suggests a rapid flow of cells into the germinal centres. It was proposed that these cells were following a normal homing pathway to the bursa-dependent areas of the spleen, of which the germinal centres are an integral part (Durkin et al., 1971).

The correlation between the localisation of the antigen and the homing of bursa cells is not clear though it is known to be unaffected by immunisation (Durkin and Thorbecke, 1973).

To examine further the kinetics of bursa cell entry into antigen-containing germinal centres an experiment was performed in which labelled bursa cells were injected at various time intervals after a single priming dose of HSA. Comparative localisation of both antigen and cells was followed by a combined autoradiography-immunofluorescence technique which enabled the observer to see simultaneously the labelled bursa cells and fluorescein-labelled HSA in cryostat sections of chicken spleen.

Fourteen, six-week old birds were divided into seven groups as in Table 6.5.1. The four test groups A, B, C and D, received a single intravenous injection of 1 mgm HSA in saline plus 5×10^7 autologous labelled bursa cells and the three control groups received HSA above plus a sham bursectomy, HSA alone, or cells alone respectively. Birds were killed either 2, 4, 6 or 8 days after immunisation and always exactly 24 hours after injection of autologous cells.

Table 6.5.1. Experimental Procedure

	Group	Antigen Day	Bursa Cells Day	Killed Day
	A	0	1	2
Antigen +	B	0	3	4
Cells	C	0	5	6
	D	0	7	8
bursectomy + antigen alone	E	0	-	6
antigen alone	F	0	-	6
cells alone	G	-	1	2

6.5.2. The twenty-four hour homing pattern of bursa cells injected into previously immunised birds

6.5.2.A HSA day 0 + Bursa cells day 1

Cryostat sections of the spleens of birds killed 2 days after an injection of HSA and 24 hours after injection of labelled autologous bursa cells showed the localisation of HSA on the surface of dendritic cells in the white pulp areas surrounding the Schweigger-Seidal sheaths (the periellipsoidal zone or PEZ). An average of four germinal centres were seen on each section but contained no HSA-bearing dendritic cells.

Labelled bursa cells were found located throughout the spleen with the greatest number also in or near the periellipsoidal zone. A small

proportion (2%) of these cells could be seen in the germinal centres. It was also found that numbers of these cells appeared in areas usually associated with germinal centre formation i.e. at the point of bifurcation of the penicillary from the central arterioles, even in the absence of histologically evident germinal centres.

6.5.2.B HSA day 0 + Bursa cells day 3

HSA-bearing dendritic cells could be seen scattered through the white pulp. Small ill-defined clusters of dendritic cells could be seen at the forks of central and penicillary arterioles. These were an average of two small germinal centres per section, which contained both HSA-bearing dendritic cells and a small number of lightly labelled bursa cells.

6.5.2.C HSA day 0 + Bursa cells day 5

At six days most antigen-bearing dendritic cells were found in large, brightly fluorescent germinal centres, which also contained many labelled bursa cells (16%). These cells were dispersed amongst the dendritic cells so that most dendritic cells were separated from each other by bursa cells. Again many labelled cells were found in the peri-ellipsoidal zone (56%).

6.5.2.D HSA day 0 + Bursa cells at day 7

At 8 days after antigen roughly one third of the germinal centres seen in cryostat sections contained HSA-bearing cells. However, all germinal centres contained many labelled cells (24%) both lightly and heavily labelled. Bursa cells were still found in large numbers at the peri-ellipsoidal zone but to a lesser degree than in

earlier groups.

6.5.2.E The localisation of HSA in sham bursectomised birds

Two-thirds of the germinal centres seen in this group were positive for HSA. Nearly all HSA-bearing dendritic cells were located within germinal centres. No labelled cells were found.

6.5.2.F The localisation of HSA in normal birds

75% of the germinal centres in this group were positive for HSA. No labelled cells were found.

6.5.2.G The 24 hour homing pattern of bursa cells into normal birds

Many labelled cells were found scattered through the spleen; 13 - 21% of the labelled cells appeared in the peri-ellipsoidal zone and 48 - 56% were located in germinal centres. No HSA-fluorescent cells were seen.

The number of germinal centres per spleen section, the number positive for HSA, for labelled bursa cells, or both is given in Table 6.5.2.

The distribution of labelled bursa cells among the various anatomical compartments of the spleen in summarised in Table 6.5.3.

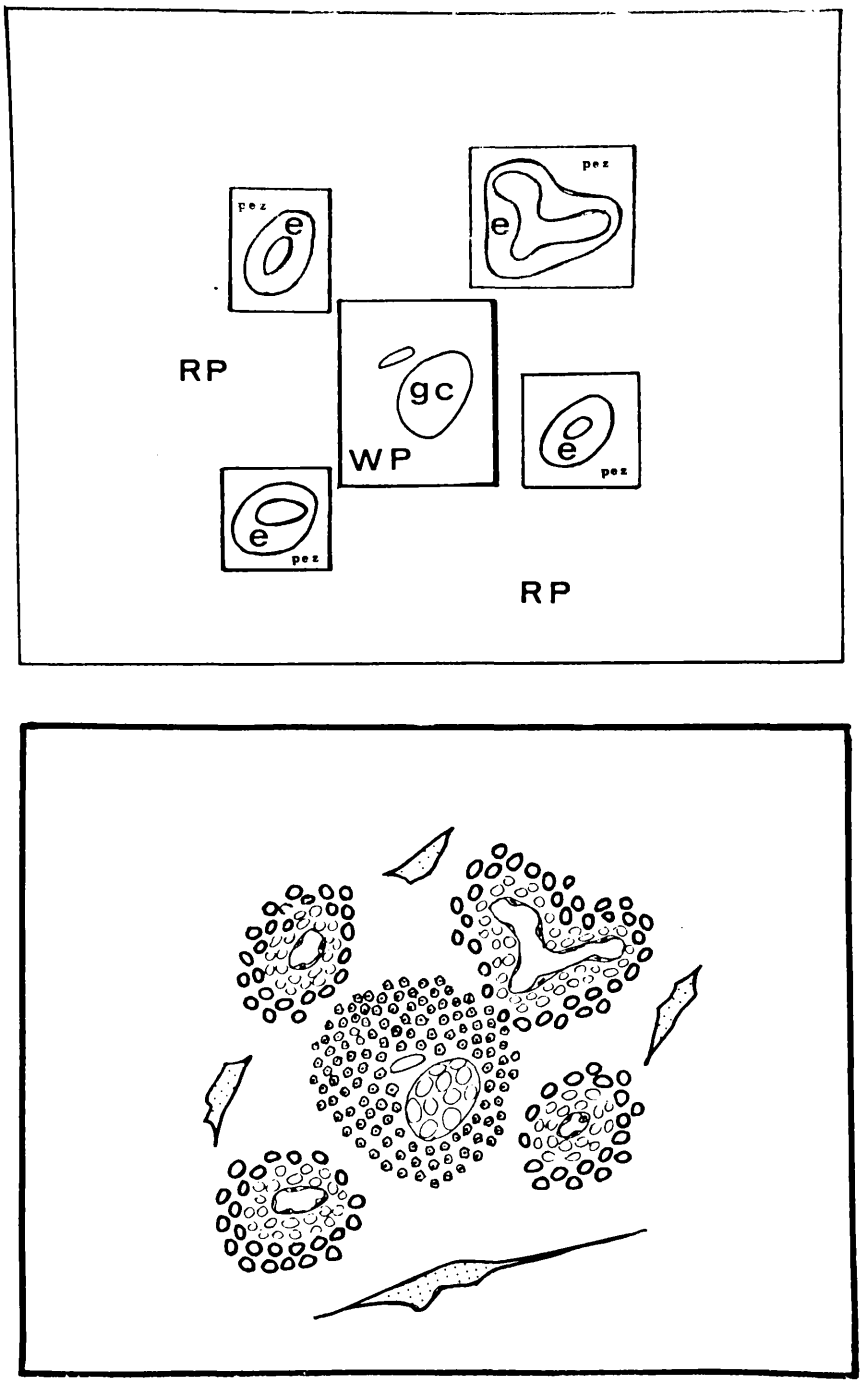


Fig. 6.5.1. Diagrammatic representation of anatomical micro-compartments of the chicken spleen.

RP = Red pulp WP = White pulp
GC = Germinal centre
E = Ellipsoid
PEZ = Peri-ellipsoidal zone

Table 6.5.2 Germinal centre formation in the chicken after a single i.v. injection of 1 mgm HSA followed by 5 x 10^7 ^{3}H-adenosine labelled bursa cells

Group	antigen	bursa cells	total no. of germinal centres	no. containing HSA	no. containing labelled bursa cells
A	day 0	day 1	ND	ND	ND
	0	1	4	0	3
B	0	3	2	2	2
	0	3	2	2	2
C	0	5	9	9	9
	0	5	5	ND	ND
D	0	7	9	2	9
	0	7	8	3	7
E	0	-	10	9	-
Ag alone + Bx	0	-	39	23	-
F	0	-	17	9	-
Ag alone	0	-	10	10	-
G	-	0	10	-	8
cells alone	-	0	16	-	16

These results show that:-

a) Labelled bursa cells home to germinal centres in a non-specific manner. In animals receiving only bursa cells (Group C) nearly 100% of germinal centres contained labelled bursa cells. In Group A, even in the absence of detectable antigen three out of four germinal centres contained labelled cells. The number of germinal centres containing HSA increased in the spleens of birds in Groups B and C, and again these all contained labelled cells.

b) Even when labelled cells are given 7 days after antigen, at a time when germinal centres containing that antigen are large and well-formed (Henderson, 1975), labelled cells are able to enter these centres, and indeed form the major proportion of the contained lymphoid cells. This suggests that the formation of a germinal centre is not an exclusive process involving only a few cells agglutinating and expanding to form an enclosed ball of cells. Rather, it is envisaged that the localisation of antigen bearing dendritic cells in a prospective germinal centre site is associated with a continuous traffic of bursa cells through this region, so that bursa cells may be continually added to a growing germinal centre.

c) The presence of large amounts of protein antigen in the spleen seems to retard the traffic of bursa cells through this organ as can be seen from the numbers of labelled cells counted in different micro-compartments of the spleen (table 6.5.3 / fig. 6.5.1.).

Table 6.5.3. Percentage distribution in splenic micro compartments of in vitro ^{3}H-adenosine labelled autologous bursa 24 hours after intravenous injection.

Group	Antigen	Labelled cells	Splenic Microcompartments RP	PEZ	E	WP	G.C.
A	day 0	day 1	28	53	0	14	2
B	day 0	day 3	38	50	0	13	0
			20	50	4	19	7
C	day 0	day 5	13	54	4	13	16
			4	60	0	30	6
D	day 0	day 7	12	41	1	22	24
			16	52	1	26	4
G cells alone	-	day 1	13	13	1	26	48
			13	21	6	11	56

RP = red pulp E = ellipsoid (Schweigger-Seidal sheath) PEZ = peri-ellipsoidal zone

WP = white pulp GC = germinal centre

In animals given labelled cells alone the majority of cells (70%) were found in the white pulp areas with approximately 50% of these inside germinal centres. This contrasts with 13% in the red pulp and 17% in the peri-ellipsoidal zone. In the animals given antigen and labelled cells the homing pattern was considerably altered. In these birds the majority of cells were found in the red pulp and peri-ellipsoidal zones whereas the white pulp numbers were considerably reduced. At early times after antigen (Groups A and B) roughly 80% of the cells were found in the PEZ (50%) and red pulp (30%) and only 16% in the white pulp. At 6 days after HSA the PEZ and red pulp contained 65% of the cells and the white pulp 32% - at 8 days after HSA the red pulp (14%) and PEZ (46%) contained 60% of the counted cells as opposed to 38% in the white pulp.

The known pattern of antigen localisation to the ellipsoids of the spleen may account for the large number of cells found in this region in animals given antigen as opposed to the control group. As the antigen moves towards the germinal centres there seems to be a progressive shift of the labelled cells towards the white pulp areas and specifically the germinal centres so that, except for the high numbers of cells still associated with the PEZ the day 8 picture is approaching that of the control group. How antigen exerts this slowing effect on lymphocyte traffic is not clear but perhaps the presence of antigen-antibody complexes in or on cells around the ellipsoids inhibits the movement of lymphocytes passing through the same region.

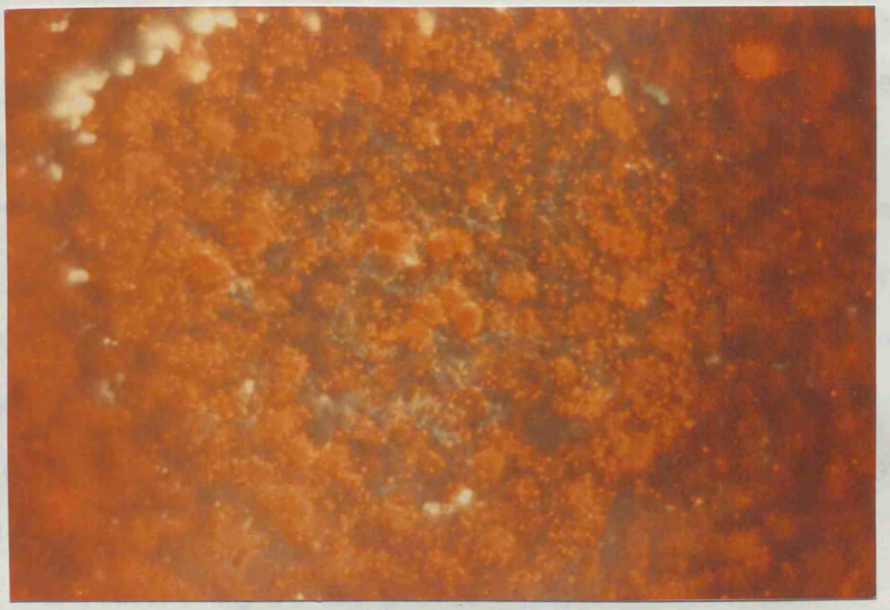


Fig. 6.5.2.

Combined fluorescence micrograph and autoradiograph. Frozen section of chicken spleen 6 days after intravenous injection of 10 mgm HSA and 24 hours after the intravenous injection of 5×10^7, ^3H adenosine-labelled autologous bursa cells. Section stained with FITC-labelled sheep anti-HSA antibody followed by processing for autoradiography. The micrograph demonstrates the presence of HSA (shown as apple green) on the surface of dendritic cells interspersed with labelled bursa cells (shown by overlying red silver grains) within the circular outline of a germinal centre.

x 720

6.6.1. Isolation of germinal centres from the chicken spleen

Various aspects of germinal centre function have been described in the preceding sections. The distribution, antigen localisation patterns, lymphocyte migration patterns, and antigen-lymphocyte interactions of germinal centres of the chicken have been discussed. Despite this information and despite almost a century of investigation the exact nature of these structures, their origin and function, remains unclear. It is not known, for example, whether germinal centres are purely temporary structures formed de novo in response to antigen or else more permanent features of lymphoid tissue anatomy. Neither is it known with any certainty what becomes of the progeny of the rapidly dividing cells within the germinal centres.

One of the reasons why it has proved difficult to define precisely the parameters of germinal centre formation has been the failure to study these structures in vitro, as independent isolated units. Fagreus in 1948 and subsequently Thorbecke and Keuning in 1956 developed techniques for the microdissection of rabbit spleen into red and white pulp fragments. Included in the latter were occasional germinal centres but these were surrounded by many other follicular components so it was difficult to make conclusions about the role of the germinal centres. Other studies have relied upon three dimensional analysis of consecutive histological sections of either lymph node (Yoke Min Sin, 1972) or spleen (Milliken, 1969).

During routine fixation of small (1 mm^3) pieces of chicken spleen for electron microscope studies it was noted that structures resembling germinal centres could sometimes be seen attached to arterial vessels, and could, with some manipulation, be detached from the surrounding pulp tissue. Based on this observation a method was devised for the

isolation and *in vitro* study of germinal centres from the chicken spleen.

Six week old White Leghorn chickens of both sexes were used. To ensure a maximum number of germinal centres in the spleen some birds were immunised 5 days prior to kill with 10 mgm human serum albumin (HSA) in 1 ml saline i.v.

All birds were killed by an intravenous overdose of sodium pentabarbitone and the spleens removed and placed in a plastic petri dish containing 5 mls of ice-cold Eagle's medium. Using fine forceps and a sterile needle the splenic capsule was gently peeled away from the spleen mass. The pulp tissue was then gradually teased out into the surrounding medium, as in the preparation of a cell suspension, leaving the arterial tree as a branched structure, easily visible to the naked eye, with numerous clumps of red and white pulp still clinging to the vessels. The arterial tree was then transferred to a petri dish of medium and further gentle teasing or combing carried out on the vessels to remove adherent pulp fragments. After further transfers to fresh medium and further "combing" the arterial tree could be seen as an elegant, pale-white, branched structure, devoid of attached pulp tissue, floating in medium.

The petri dish was then transferred to the stage of a light microscope and the arterial tree observed under the 4x and 10x objectives. The branches of the tree have a striking appearance under the microscope and can best be observed with the light diaphragm almost closed. The germinal centres were easily visible at the forks made by centres

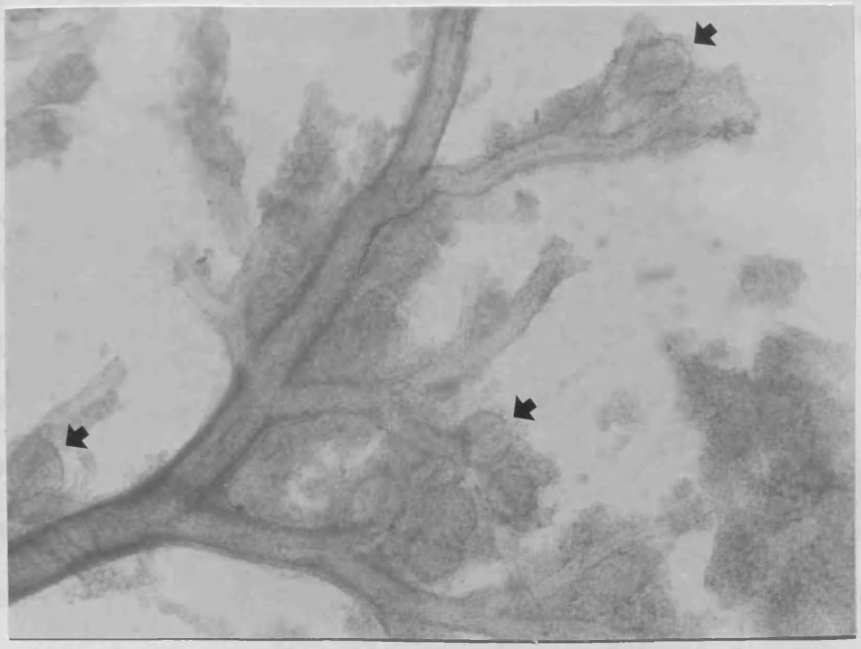


Fig. 6.6.1.

Photomicrograph of a segment of the arterial tree from a chicken spleen during the germinal centre isolation procedure. Sitting in the forks made by central and penicillary arterioles are numerous germinal centres (arrowed), seen here as pale, round, spheres. There are 13 centres in this photograph.

x 120

and penicillary arterioles as large,spherical balls attached to the vessel walls, giving the appearance of "fruit" on a tree (Fig. 6.6.1.) They varied considerably in size, with an average diameter of approximately 150 μ. The centres possessed a certain degree of elasticity as was shown when a cover slip was introduced into the tissue preparation so as to lie over the germinal centres. Downward pressure exerted on the glass cover slip resulted in a flattening of the centres. Release of pressure was followed by an immediate return of the centres to their original spherical shape.

The germinal centres were enclosed in what appeared to be a thin connective tissue capsule, arising from the adventitia of the associated arteriolar vessels in such a way that the capsule forms an integral part of the vessel wall. It was possible, using very fine forceps to cut off individual centres from the arterial tree, but such centres were invariably accompanied by small fragments of attached vessel.

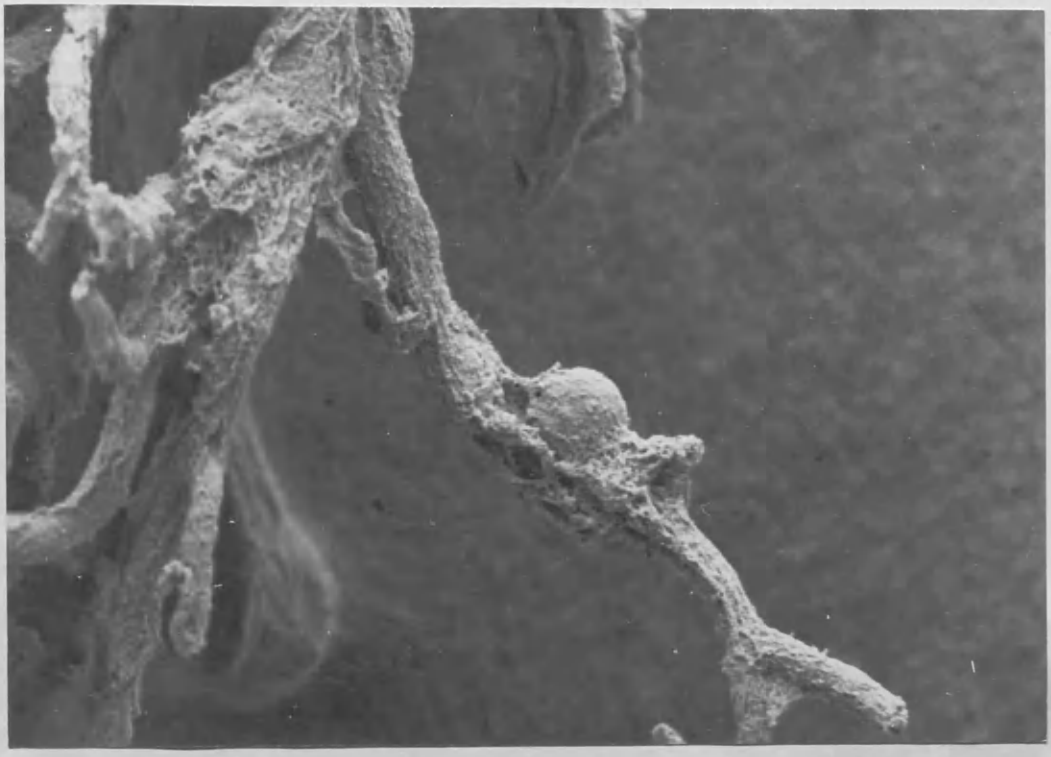
Scanning E.M. studies on isolated germinal centres

In an attempt to gain further insight into the spatial aspects of germinal centres a brief scanning electron microscope study was performed on some of the preparations resulting from the above experiment. The isolated centres and their associated vessels were fixed in formol saline, placed on a block,subjected to critical point drying, coated with a layer of vanadium, and viewed with a Phillips SM 3000 Scanning Electron Microscope. The germinal centres were easily identified, both by their shape and positioning at the points

of bifurcation of the penicillary with the central arterioles (Fig. 6.6.2., 3 and 4). The spherical appearance of the centres had been retained during the fixation procedure giving an unusual three-dimensional aspect to those structures, so often viewed in histological sections two-dimensionally. The surface of the germinal centre in Fig. 6.6.4. appeared smooth, with no signs of an open reticular meshwork through which cells may pass. Several small polymorphic objects were noted on and adhering to the surface of the germinal centre but it was difficult to make out whether these were attached small cells or connective tissue debris.

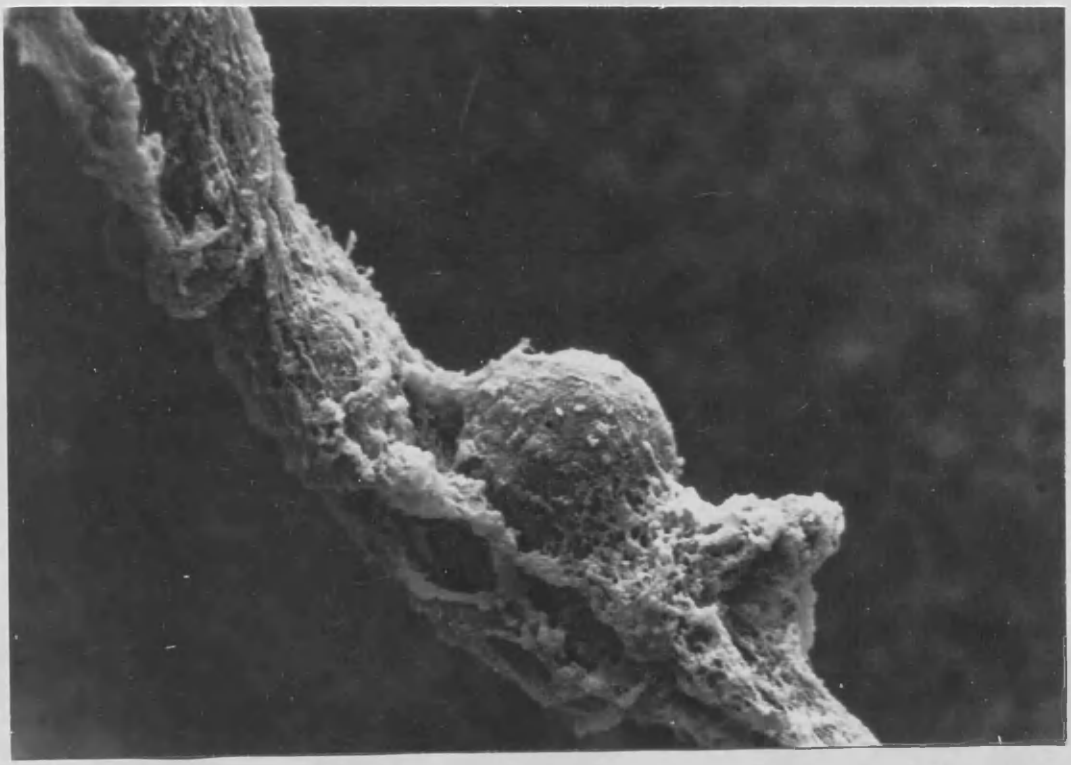
This is the first report of an in vitro study of intact isolated germinal centres at either the light microscope or Scanning E.M. level. The positioning and appearance of these structures makes it difficult to accept that they represent a simple transient cellular aggregation which appears in response to antigenic stimulation. It is far more likely that the germinal centre environment is a specialised permanent area in lymphoid tissue which only becomes histologically recognisable with accumulation and division of certain lymphoid and non-lymphoid cells.

Fig. 6.6.2.



x 120

Fig. 6.6.3.



x 250

Figs. 6.6.2. and 3.

Scanning electron micrographs of fragments of the arterial tree of a chicken spleen. The distinctive, spherical shape of a germinal centre can be seen sitting above the fork of two vessels.

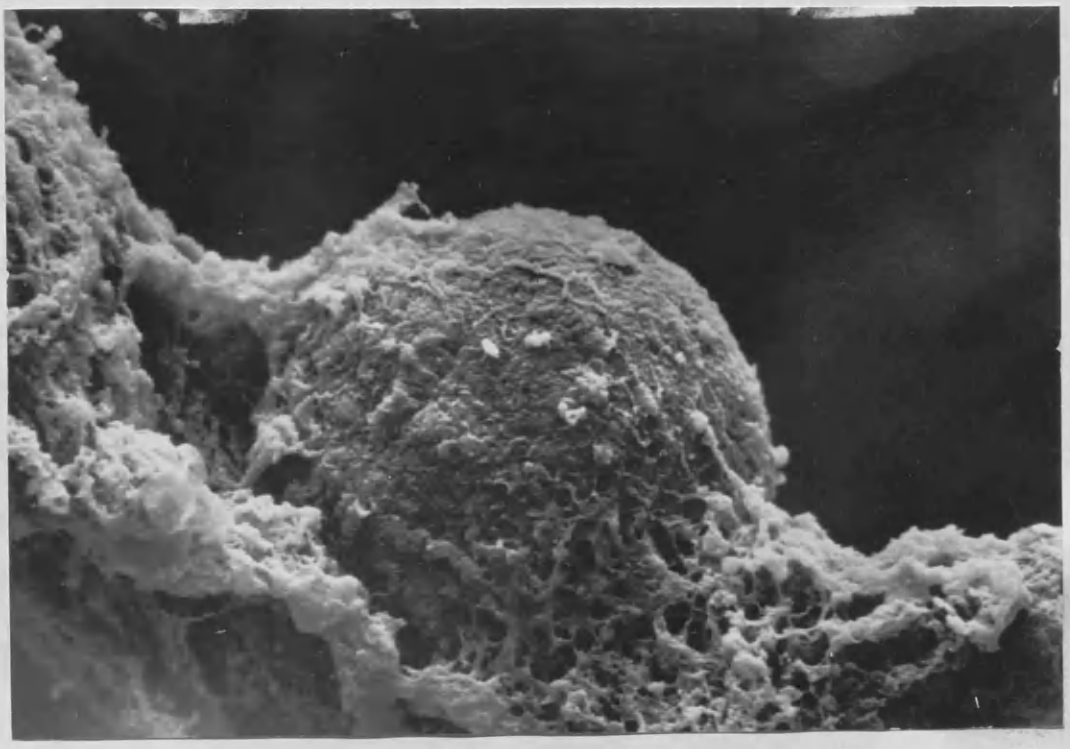


Fig. 6.6.4.

Scanning electron micrograph of germinal centre of chicken spleen. The centre is attached to a small blood vessel running from left to right and is held by strands of connective tissue at the lower edges. The surface of the centre is fairly smooth though there are small collaginous fibres running over it and occasional small round objects projecting above the sphere. These may represent small cells (5μ) but might also be connective tissue debris.

x 625

7. Fate of an incorporated protein antigen (HSA-I^{125}) in Freund adjuvant emulsions injected into the breast muscle of the chicken

The development of germinal centres in local lesions caused by Freund's complete or incomplete adjuvant emulsions has been described in an earlier section (1.1.). Whereas few germinal centres were found after one week in FCA lesions many large, well-formed centres were seen in the FIA granulomas throughout the period of study. These structures were identical to splenic germinal centres in their size, shape, and staining characteristics. For example, staining with fluorescein-labelled antibody to chicken 7S immunoglobulin revealed the classical pattern of dendritic cell surface immunoglobulin within these centres.

It was felt that their presence (in the case of FIA) and absence (in the case of FCA) in local lesions was closely linked to the adjuvant effect exerted by the complete and incomplete emulsions on an incorporated protein antigen such as HSA. Circumstantial evidence pointed to such a role but no direct proof had been found, from immunofluorescence studies, linking the antigen to the germinal centres i.e. no HSA had been observed on dendritic cells within these local centres, as is the case in splenic germinal centres after an intravenous injection of antigen.

This failure was attributed to the small quantity of antigen normally incorporated in these emulsions (40μgms) as only a tiny fraction of this antigen is available for germinal centre localisation and is probably below the threshold of sensitivity of the fluorescent antibody technique.

Consequently an experiment was set up using I^{125}-labelled HSA

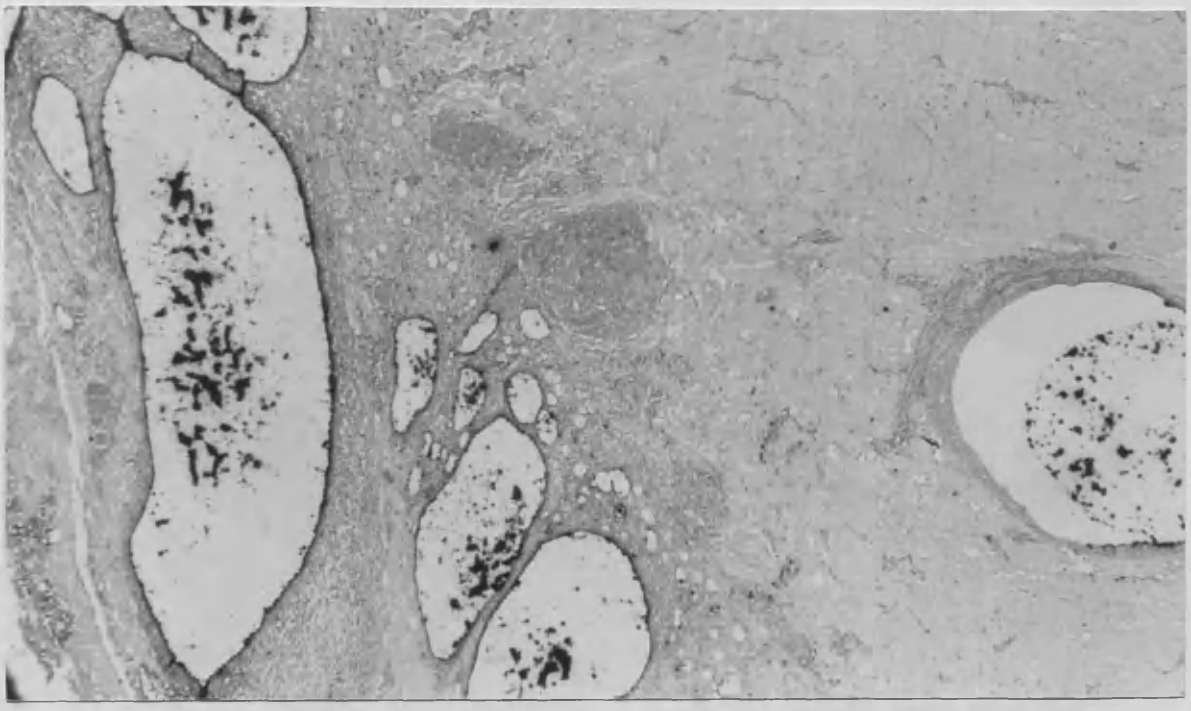


Fig. 7.1. Autoradiograph of section of chicken breast muscle one week after the local injection of FCA containing HSA-I^{125}. The antigen is easily visible as black deposits in large oil vacuoles. Smaller deposits are visible in the surrounding granulomatous tissue.

x 120 U.P.

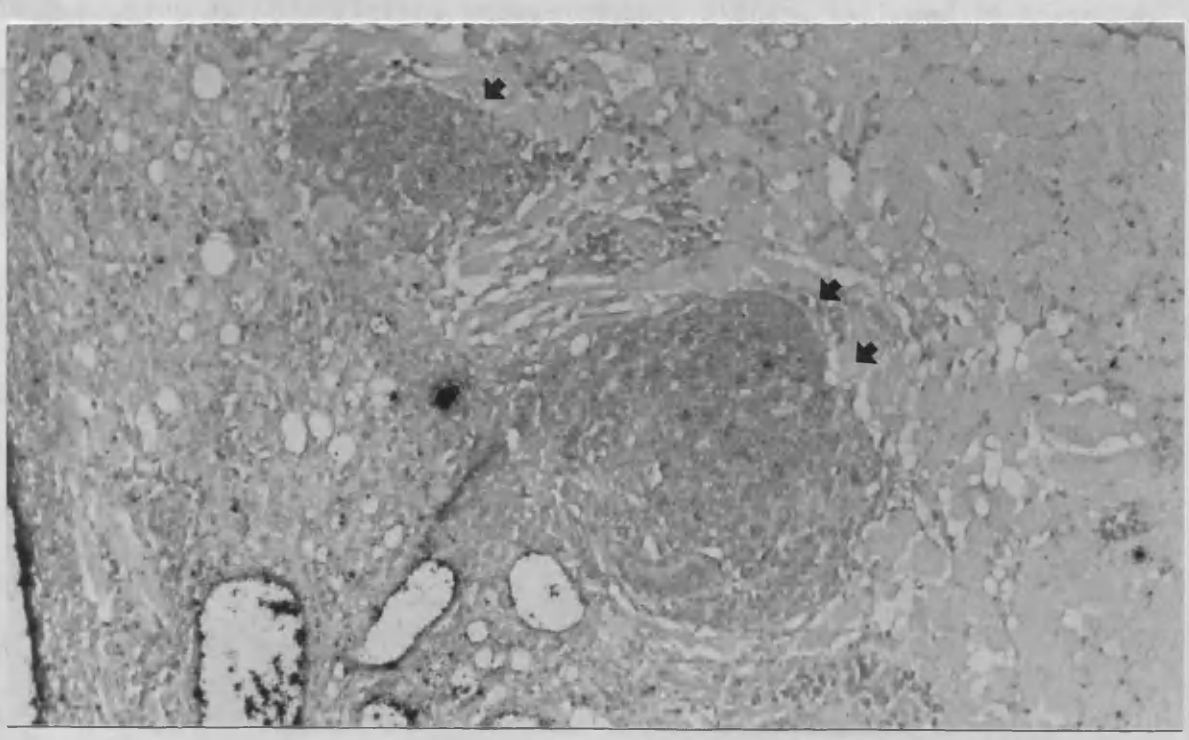


Fig. 7.2. Two aggregates of lymphoid cells (arrowed) from Fig.7.1. above were considered to represent distorted germinal centres. To the bottom left are scattered antigen-containing oil vacuoles. To the right is unaffected muscle tissue.

x 300 U.P.

incorporated in both FCA and FIA emulsions. Autoradiography is thought to be 100-1000 times more sensitive than immunofluorescence and as such might detect the very small quantities of antigen involved.

Two groups of birds were injected intra-muscularly with 150µgms of HSA-I^{125} in either FIA or FCA. Birds from both groups were killed at one, two, and four weeks after injection, and tissue sections of the spleens and granulomas processed for autoradiography.

Histologically the lesions were identical to those described in experiment 1. FCA resulted in a bulky epithelioid granuloma, with an intense proliferation of epithelioid cells in thick, pallisaded layers around and between oil vacuoles. FIA on the other hand gave rise to an inconspicuous lesion confined to the intermuscular septae, consisting of a thin layer of fibrous material enclosing numerous oil vacuoles, interspersed with clumps of macrophages and dense lymphoid areas running as thin strands between muscle fibres. Included in these lymphoid areas were many compact germinal centres (Fig. 7.4.).

Examination of autoradiographs of spleen and granuloma tissue sections revealed subtle differences in the handling of the HSA-I^{125}.

a) Spleen sections. In the spleens of both FCA and FIA treated animals HSA was present, as demonstrated by overlying silver grains, wholly within germinal centres in the white pulp. Both groups of animals had large numbers of HSA-containing germinal centres with the greatest number at one week and a gradual drop in numbers to the fourth week. This paralleled a drop in the intensity of silver grain deposition over the centres , suggestive of a gradual diminution of the amount of radioactive HSA present. No difference was noted in the morphology of

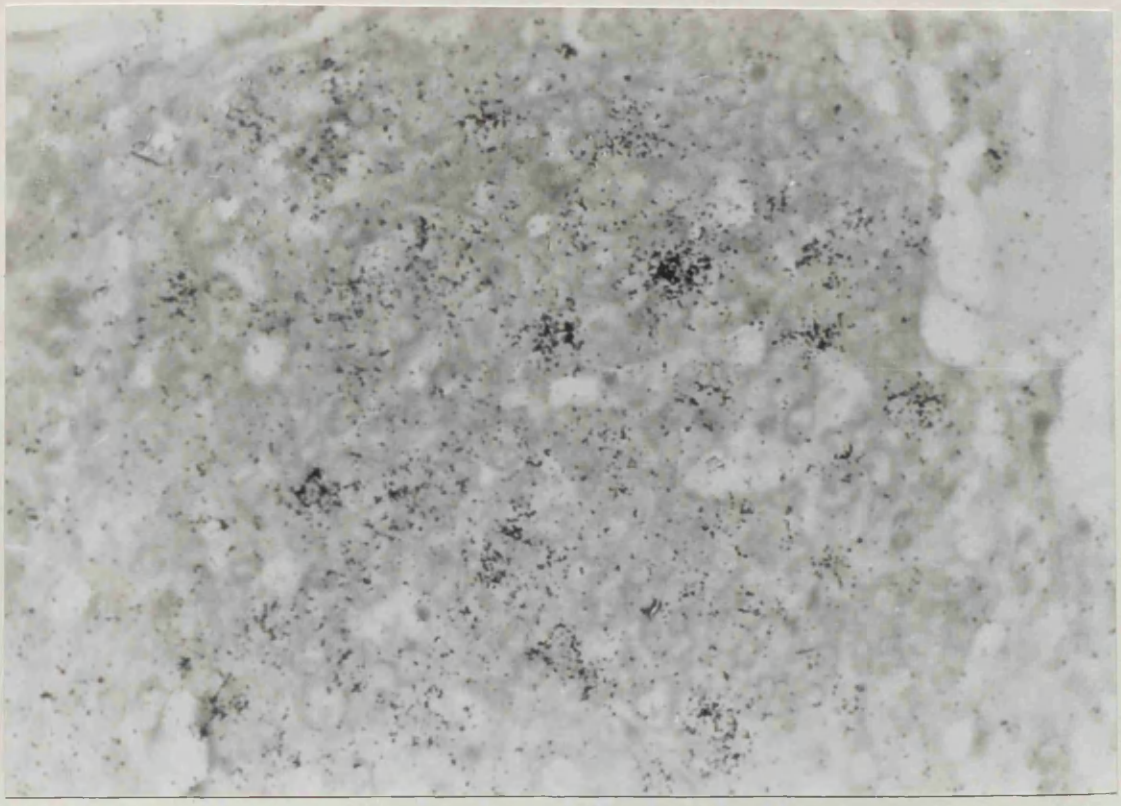


Fig. 7.3. Closer examination of the larger lymphoid cell aggregate in Fig. 7.2. reveals a typical germinal centre pattern of antigen-bearing dendritic cells similar to that normally seen in the spleen.

x 3000 U.P.

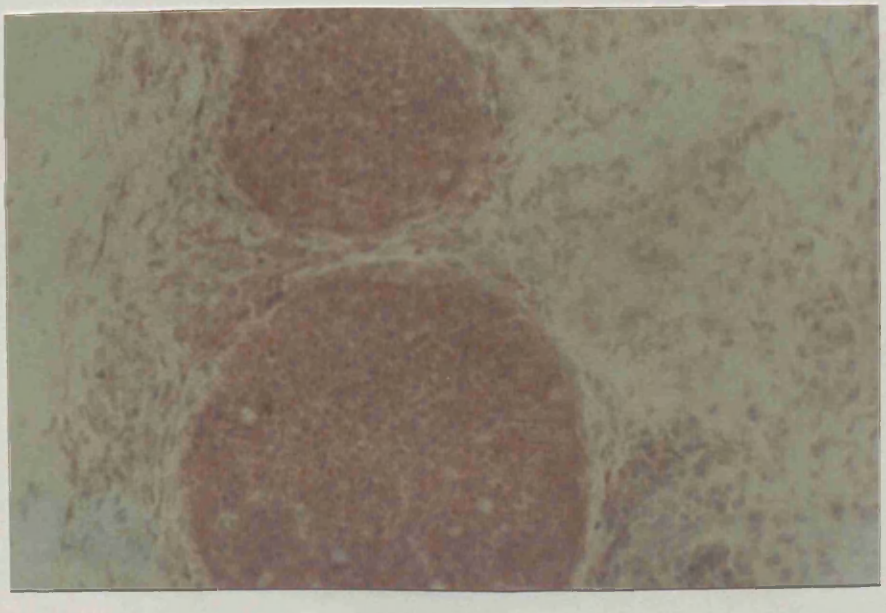


Fig. 7.4. Autoradiograph of section of breast muscle four weeks after a local injection of FIA and HSA-I^{125}. Seen here are two large, compact, well-formed germinal centres lying in a strand of fibrous tissue.

x 180 U.P.

the morphology of the germinal centres between the two groups and the antigen localisation pattern was of the classical dendritic cell surface type (see Fig. 6.2.7.).

b) Granuloma sections: Heavy deposits of HSA were visible in the FIA lesions throughout the time period studied. These were mostly confined to the numerous oil vacuoles present though individual macrophages with intracellular antigen were also present, in the areas between vacuoles. Also visible were many antigen-bearing cells with a surface antigen pattern contrasting with the intracellular pattern just described. These cells were tentatively identified as dendritic cells because identical cells were present in the local germinal centres, with a fine surface reticular pattern of silver grains (Fig. 7.4.). The antigen localisation pattern of these centres closely resembled that of the splenic germinal centres. This pattern was similar at one, two, and four weeks after injection.

In the FCA granulomas heavy deposits of antigen were again seen in oil vacuoles throughout the study period (Fig. 7.1.). Antigen was also visible in macrophages and on the surface of dendritic cells in the surrounding areas. No germinal centres were seen in the two week and four week lesions. At one week however, several bizarre,lymphoid nodules were present (Fig. 7.2.) which on closer inspection (Fig. 7.3.) revealed a dendritic cell pattern of localised antigen. It was thought that these structures represented distorted or damaged germinal centres. The normal clear-cut,circular outline and sharp demarcation from the surrounding tissue was lost and the outline highly irregular.

This experiment confirmed that local germinal centre formation was involved in the handling of antigenic material as silver grains

corresponding to the HSA-I^{125} were clearly seen in these centres, at least in FIA animals. Early FCA granulomas contained abnormal germinal centres which also displayed HSA localisation. Very little difference was observed in antigen processing in the spleens of the two groups. Both contained antigen localised to dendritic cells in germinal centres which persisted up to the fourth week.

It would appear from this experiment that a dual immune response to the injected antigen takes place. The spleen responds, but so also do the lymphoid tissues of the newly-formed local granuloma. One result of this immune response is the formation of antigen-containing germinal centres which persist for at least four weeks, and possibly longer, in the spleens of both groups of birds and in the granulomas of the FIA group. In the FCA group distorted though still recognisable antigen-containing centres were present at one week but rarely seen thereafter. The presence of mycobacteria in the adjuvant emulsion in some way interferes with germinal centre formation in the granulomas, but not the spleens, of the FCA group.

DISCUSSION

DISCUSSION

In the first part of this thesis an attempt was made to gain greater insight into the conditions governing the successful use of Freund-type adjuvant emulsions in the chicken. Interest centered on the formation of a granuloma at the site of adjuvant injection and the interaction of the adjuvant-emulsion with the various populations of cells which form such a granuloma, particularly in regard to their role in the potentiation of the antibody response. A comparison was made between the effects of the antigen in the incomplete adjuvant (FIA) and the complete adjuvant (FCA) on the histopathology and antibody response of the recipients.

Choice of Experimental Model

The choice of experimental animal, the chicken (Gallus domesticus) was based on several factors. It had been shown previously that injection of Freund's complete adjuvant into the pectoral muscle of the chicken resulted in the formation of a large and striking local granuloma (White and Marshall, 1964). This site was particularly suited to this study as the granuloma thus formed was contained within the boundaries of the injected muscle. This had two advantages. Firstly the histological picture of the muscle tissue was relatively simple compared to the lymphoid nature of the granuloma so it was easy to define the extent of the granulomatous infiltration. Secondly the granulomatous response seldom, if ever, spilled over to the opposite, uninjected, pectoral muscle so this was suitable as control tissue.

This model had been analysed further by several groups (Steinberg et al., 1970; French et al., 1970) and various parameters of the response had been described in detail. In addition this laboratory has considerable experience in the problem of antigen localisation in the chicken and detailed knowledge was available concerning the kinetics of the response to HSA (White et al., 1970; White et al., 1975). This was an important part as I wished to investigate possible alterations in the handling of antigen which occur with use of adjuvants.

Finally the chicken has the advantage of possessing germinal centres which have a very distinctive appearance. The original hypothesis (White, 1973) suggested an effect of the adjuvant on germinal centres, so the circular, compact, uniform, appearance of chicken germinal centres was especially suitable for this purpose.

Effects of Freund's Adjuvants

In attempting to explain the mode of action of those adjuvants named after him, Freund (1956) suggested that their activity rested principally on three separate phenomena:

a) The establishment of a portion of the antigen in the form of a depot at the site of injection making possible a gradual and continual release of antigen for stimulating antibody synthesis.

b) The provision of a vehicle for transport of emulsified antigen throughout the lymphatic system to distant sites, such as the lymph nodes and spleen where new foci of antibody production can be established (Freund, 1951; Rist, 1938).

c) The formulation and accumulation of cells of the mononuclear series which are appropriate to production of antibody at local and distant sites.

Freund concluded however that the exact mechanisms were not known. Work carried out during subsequent years has not significantly altered these basic concepts to any significant degree.

Effect of Freund's Adjuvants on the Chicken

The histopathological results of administering FCA and FIA to the chicken follow the same broad pattern as in other species, but have additional features which are peculiar to this species. The initial event for both types of adjuvant is the formation of a local granuloma at the site of injection.

In the case of FIA the foreign body,(low turnover) granuloma is surprisingly inconspicuous at post-mortem. The tissue reaction is confined to an enclosure of oil vacuoles by a thin layer of fibrous tissue. This presents as a barely detectable thickening of the fibrous strands between muscle bundles or over the surface of a separate muscle. (see Fig.1.2.a.). In addition scattered clumps of macrophages can be located, particularly if a small quantity of indian ink was added to the saline phase of the injection mixture (see Fig.1.2.b.). Segregated away from these macrophage-fibroblast granulomatous areas, with their oil vacuoles were dense lymphoid areas usually running as thin strands sandwiched between muscle fibres deeper in the substance of the muscle than the oil vacuoles. Part of such lymphoid areas consisted of packed small and large lymphocytes but within such areas were many oval germinal centres, adjacent to small arterioles running through the muscle tissues. These ovoid or spherical collections of lymphocytes could be definitely identified as typical germinal centres in frozen sections stained with fluorescein-labelled anti-chicken 7S

immunoglobulin (Fig.1.2.4.) or by the grain pattern of scattered antigen-bearing dendritic cells following the preparation of autoradiographs after injection of I^{125}-labelled HSA in water-in-oil emulsion. The resulting pattern of immunoglobulin or labelled antibody was identical with the pattern of dendritic cells throughout a splenic germinal centre.

In contrast the lesion which develops at the site of injection of FCA is a bulky, epithelioid cell (high turnover) granuloma, which shows an intense proliferation of epithelioid cells in thick pallisaded layers around and between oil vacuoles. In established granulomas at 4 weeks and later, although lymphoid areas can be identified, it is difficult to locate any typical germinal centres. The morphological appearance of germinal centres in birds injected intramuscularly with HSA in FIA is clear-cut and easy to recognise. The germinal centres in the centre or periphery of the granuloma which develops after injection of HSA in FCA are not only relatively scanty but difficult to identify with certainty as germinal centres. The normal clear-cut circular outline and sharp demarcation from the surrounding looser lymphoid tissue is lost and the outline may be highly irregular. Such distorted germinal centres are commoner in the early granuloma at 1 and 2 weeks after injection, and often could not be identified with certainty. (Fig. 1.2.k.). However, autoradiographs prepared from local granulomata which were the result of injecting I^{125}-labelled HSA in FCA showed that such distorted centres displayed the distinctive antigen-dendritic cell pattern which typifies normal germinal centres in the spleen (fig. 6.2.7.).

Other findings in the histopathology section followed the patterns of previous investigations(Freund 1956). There was considerable retention of the adjuvant mixture at the injection site and the protein antigen, the mineral oil and the mycobacteria could all be seen draining into the local lymphatics.

The serum antibody responses followed the patterns described in detail by French and her colleagues (1970) with the FCA causing a secondary rise in antibody to the HSA peaking at about 8 weeks at a level of 10 - 100 times the normal primary response. Using immunofluorescence techniques it was confirmed that while the spleen was largely responsible for the primary antibody response the secondary response was almost totally the contribution of the local granuloma. No anti-HSA plasma cells were visible in the spleen after the first week, (with the exception of occasional antibody centres - fig. 1.2.s.), in contrast to the granuloma which became increasingly populated with plasma cells, many of which were actively engaged in synthesis of antibody to the antigen (HSA) incorporated in the adjuvant mixture.

The 7S-serum immunoglobulin levels in FCA treated animals were also subject to an overall increase (fig. 1.2.r.) to levels considerably higher than in normal chicken serum. It was not established whether this increase was due to the synthesis of excessive amounts of specific antibody but in view of the findings of Humphrey (1963) in the rabbit it is likely that antigen-specific antibody would not account for all the excess immunoglobulin. This finding was confirmed by White and Cobb (personal communication) who found that the serum 7S immunoglobulin levels of chickens treated with FCA containing M. smegmatis

showed a considerable rise above normal over a period of six weeks after injection.

With its increased vasculature, and organisation into distinct tissue compartments,of lymphocyte infiltration, plasma cell differentiation and antibody production, the FCA granuloma at times assumed the appearance of organised lymphoid tissue such that parts of the granuloma came to resemble a kind of splenic analogue.

Impressions of this kind were noted previously by Smith and his colleagues (1970) after using FCA in sheep, and also by Spector and Lykke (1966) in the rat. The granulomatous inflammatory response to Freund's complete adjuvant seems to provide a local environment which is conducive to extensive lymphoid cell colonisation.

Experiment 2 demonstrated very little difference between the antibody responses of young (6 week) and adult birds in response to FCA. Thus the adjuvant effect is clearly not age dependent. Young birds treated with FIA did reveal a tendency to respond with higher rebound antibody levels than older birds. This was thought to be attributable to the continued presence of the bursa in the young animals, which may still be seeding immature B cells to the periphery.

The dose response characteristics of the antibody response to antigen in FCA were next explored (Experiment 3). It was clearly shown that a reduction in the amount of mycobacteria incorporated in the adjuvant, from 5 mg to 50 μgm made no difference to the response to an included antigen (HSA). If anything the lowest dose of mycobacteria produced the highest antibody response (Fig. 3.1.). In addition extracts of the granulomata from this experiment revealed an inverse

relationship between the dose of mycobacteria and antibody extracted such that the lowest dose (50 µg) resulted in the highest amount of extracted antibody. This result suggested that the active component of the mycobacteria is required in very small amounts for an effective adjuvant response. Increasing the weight of mycobacteria may possibly hinder the effectiveness of the active component.

The next section (Experiment 4) examined the effects of separation of the adjuvant components on subsequent antibody production. It was shown that it was only necessary to incorporate the antigen in the water-in-oil emulsion while the mycobacteria were effective even in saline. In addition the protein antigen component and the mycobacterial component did not have to be given in the same emulsion. The mycobacteria are equally effective if they are present at the antigen depot site as late as 7 days after injection of the antigen. This will be mentioned later with reference to the work of Zbar and his colleagues (1971).

Section 5 explored the possibility of bursa cell traffic to the granuloma as an explanation for the local antibody production. The source of the antibody-forming cell precursors was not clear so labelled thymus and bursa cells were intravenously injected and monitored for their ability to home to the granuloma. It was found that while moderate numbers of thymus derived cells appeared in the FCA granulomas (Fig.5.2/3/4) only a few bursa-derived cells could be found in the lesion (Fig. 5.1). In contrast many of the injected bursa cells were found in the spleen, both in the white pulp and within germinal centres. Those labelled bursa cells seen in the granuloma were

relatively lightly labelled compared to the original inoculum, suggesting that cell division had taken place with a consequent dilution of isotopic label. It is possible that the injected bursa cells divide in the spleen prior to seeding to the granuloma.

Conclusions regarding the formation, structure and function of germinal centres in the chicken.

The second half of this thesis examined the role of germinal centres in the immune response of the chicken in an attempt to evaluate the presence of these structures in local granulomata. Experiment 6.1. summarised the ubiquitous nature of germinal centres in the domestic fowl and indicated that they are a common and important component of both lymphoid and non-lymphoid tissues of this bird.

Experiment 6.2. re-examined the details of antigen localisation to germinal centres of the chicken spleen. The reports of White and his colleagues (White, 1963; White et. al., 1970) were confirmed. Basically, an intravenously injected protein antigen circulates in the blood until antibody is synthesised and the resulting antigen-antibody complexes then gain access at roughly 48 hours after injection to the splenic white pulp (Fig. 6.2.1.). Most of this antigen is then rapidly catabolised but a small fraction persists as complexes on the surface of large reticular or "dendritic" cells. These dendritic cells migrate through the white pulp to the point of division of the central arteriole into penicillary arterioles. Here they form clusters of cells with small lymphocytes from about 72 hours after injection. These clusters or cellular aggregates appear to grow by the progressive inclusion of dendritic cells and lymphocytes until by day 6 after injection antigen

is only visible within germinal centres (Fig. 6.2.2.) (except for occasional deposits on the walls of vessels). This antigen may persist at this site for several weeks after injection.

In the next section it was shown that this localisation process was not antigen specific, neither were the germinal centres exclusive for one particular antigen. Two protein antigens, human gamma globulin (HGG) and human serum albumin (HSA) were injected simultaneously and their course followed by a combined autoradiography-immunofluorescence technique (Henderson and Smithyman, 1974). The two proteins appeared in the spleen simultaneously, were taken up by the same dendritic cells and carried into the same developing germinal centres (Fig. 6.3.1. - 3). This indicated that the dendritic cell localisation process was not antigen specific. The antigen is carried by the cell in the form of an antigen-antibody complex* so if the dendritic cell has Fc receptors it need only recognise the Fc portion of autologous antibody. This result suggested that at least two but more probably a large number of antigens may be present in the same germinal centre (an important point to which I will return).

The next experiment was concerned with the well-known homing patterns of the thymus derived (T) cell and bursa-derived (B) cell to the chicken spleen (Durkin et al, 1970; De Sousa et al, 1973). This experiment confirmed that 50% of the labelled B cells counted in spleen sections 24 hours after injection could be seen in germinal centres (Fig. 6.4.1.). In addition many of these cells appeared to have divided (as judged by overlying grain counts). In contrast labelled thymus cells did not enter germinal centres but remained at the periphery (Fig. 6.4.2.).

* NB Aggregated but not monomeric HGG is also taken up by chicken spleen dendritic cells via the Fc portion of the human globulin.

This indicated that the small lymphocytes seen at the early stages of germinal centre formation were probably of B cell origin. The observation of specific antibody-containing cells in splenic germinal centres of adjuvant treated animals (Fig. 1.2.s.) indicates that the B cells included in such centres are immunologically competent and may develop the capacity to produce specific antibody.

The kinetics of antigen and bursa cell localisation were explored in experiment 6.5. which followed the localisation of autologous labelled bursa cells to germinal centres formed by a specific antigen (HSA). The aim of the experiment was to establish the entry time of bursa cells into a developing germinal centre, in other words, at what time the bursa-cell "passengers caught the dendritic cell train". The results indicated that bursa cells may enter established germinal centres up to at least 7 days after the administration of specific antigen (Fig 6.5.), suggesting perhaps that the route into germinal centres is not closed at this time. Not only did bursa cells gain entrance to the centres at this time, they also constituted the majority of the lymphocytes found in these structures, though it must be remembered that the intravenous administration of 5×10^7 bursa cells is hardly physiological.

The last experiments in the germinal centre section described attempts to separate out and isolate these structures from chicken spleens. The resulting isolated centres appeared to have a tough, elastic, connective tissue capsule and were intimately associated with arteriolar vessels (Fig. 6.6.1.) in the spleen. It was concluded that the germinal centre site is a permanent rather than transient anatomical structure which is subject to expansion and contraction during an immune response.

Conclusions regarding the fate of a protein antigen (HSA-I^{125}) incorporated in Freund's adjuvants.

The presence of large well-formed germinal centres at the site of FIA granulomas prompted the logical question as to whether these structures were involved in the local immune response. The third part of the results dealt with this question. Isotope-labelled HSA was injected as part of the adjuvant mixture and its distribution followed by autoradiography. The antigen could be seen in both FIA and FCA granulomas in oil depots throughout the lesions.

In addition FIA granulomas, over the four week period studied, contained numerous well-shaped germinal centres with HSA on the dendritic cells within (Fig. 7.4.). In contrast FCA granulomas did not contain any recognisable germinal centres after the first week. Even at one week curious lymphoid cell aggregates (Fig. 7.2.) could only be identified as germinal centres because of the distinctive dendritic cell antigen patterns in these areas.

Spleen sections from both groups were also monitored for antigen localisation. Curiously enough there was little evidence of disruption of antigen localisation in this organ. At one week the spleens of both FCA and FIA treated animals possessed numerous HSA-containing germinal centres. At two and four weeks both groups still possessed numerous antigen-containing centres of normal appearance. The only difference between the germinal centres at the various time periods was a slight drop in the silver grain density over the dendritic cells of the centres, suggesting a slow diminution of the antigen.

It was concluded from this experiment that local germinal centre

formation was intimately connected with the response to an adjuvant-incorporated antigen. In addition it appeared that while germinal centres formed early and in large numbers in FIA lesions, those in FCA granulomas formed only with difficulty and did not persist beyond one week. It was thought that the presence of mycobacteria in the FCA emulsion interfered in some way with the formation of the centres.

It would appear from the presence of antigen in both spleen and local lesion that a synchronous immune response takes place in both tissues. This involves the formation of antigen-containing germinal centres in both sites. In the case of the FCA treated animals however parallel germinal centre formation to that in the spleen is prevented.

Possible mechanism of action of Freund's adjuvants on antibody formation in the chicken

In addition to those criteria originally put forward by Freund in 1956 to explain the action of these adjuvant emulsions the results of this study indicate the existence of a further mechanism, first proposed by White in 1973, in which interference of germinal centre formation by mycobacteria results in a loss of feedback control of antibody biosynthesis, with the consequent production of high levels of antibody.

In attempting to explain this additional mechanism I would like to advance a slightly revised hypothesis, based on a number of facts and observations, both from the literature and from this study, which are shown here in tabulated form:

Factors involved in the use of Freund's adjuvants

	FCA	FIA
1.	Bulky epithelioid granuloma	Inconspicuous foreign body granuloma
2.	Large macrophage component	Large macrophage component
3.	Many plasma cells	Few plasma cells
4.	Large number of T cells from the circulation	Low number of T cells from the circulation
5.	Low number of bursa cells from the circulation	Low number of bursa cells from the circulation
6.	High level of antibody production in the granuloma	Low level of antibody production in the granuloma
7.	High level of circulating antibody at four weeks	Low level of circulating antibody at four weeks
8.	Granuloma abolished by bursectomy	?
9.	Plasma cells and antibody abolished by bursectomy	?
10.	Antibody response is not age-dependent	Antibody response is slightly age dependent
11.	Antibody response of granuloma inversely proportional to dose of mycobacteria	-
12.	Antibody response dependent on antigen in oil depot	Antibody response dependent on antigen in oil depot
13.	Mycobacteria must be in tissues at peak time of germinal centre formation	-
14.	High 7S immunoglobulin levels	Normal 7S immunoglobulin levels

	FCA	FIA
15.	Many antigen-containing germinal centres in spleen from 1-4 weeks	Many antigen-containing germinal centres in spleen from 1-4 weeks
16.	Few distorted germinal centres in granuloma at one week, then none	Many germinal centres in granuloma from 1-4 weeks

Role of germinal centres in the immune response

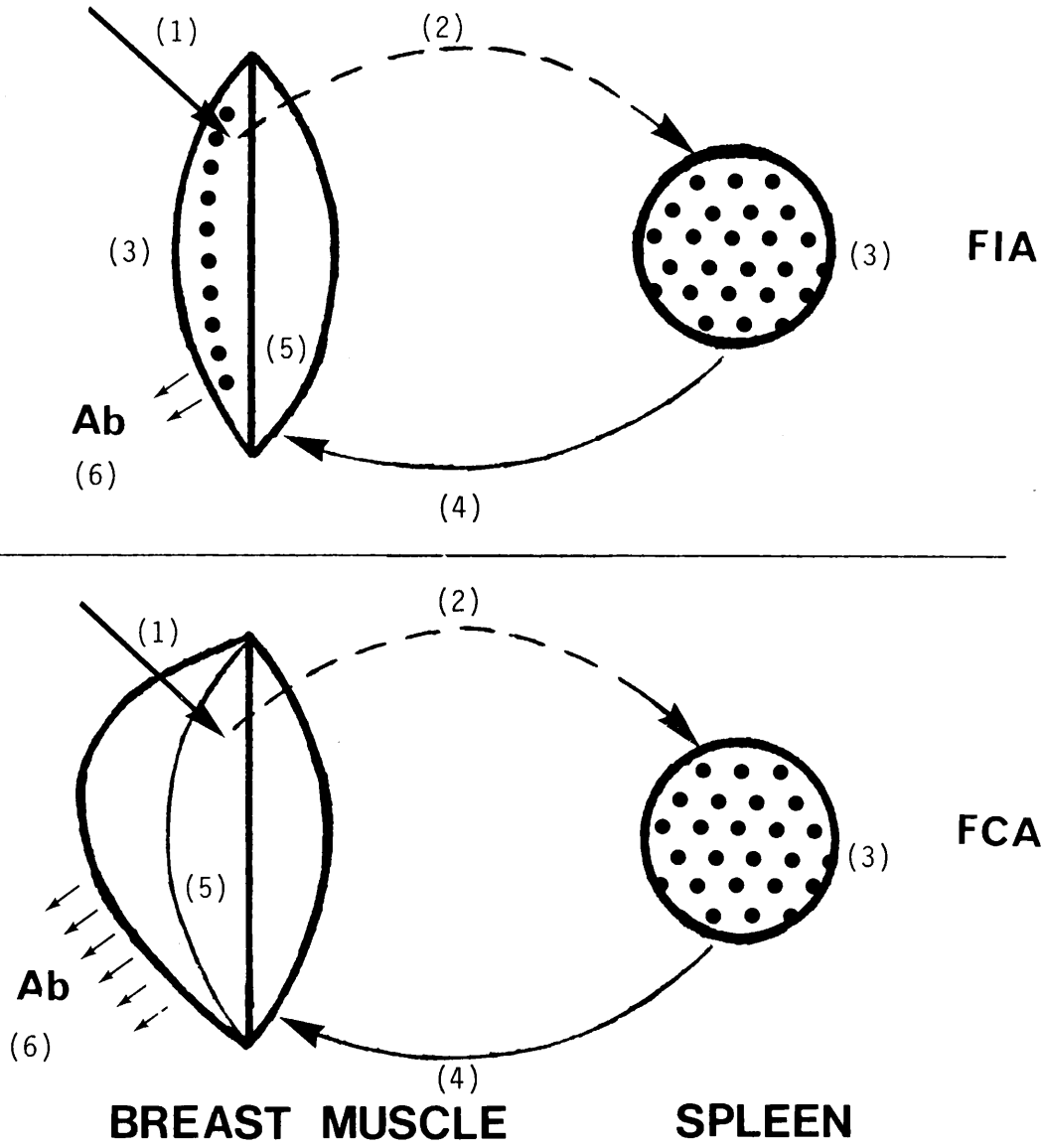
1. Expansion of a population of immunocompetent lymphocytes
2. Expansion of a population of B memory cells
3. Antigen localisation for long periods of time
4. Control of tolerance ?
5. Dependent upon histocompatibility between component cells
6. Dependent upon intact thymus or supply of T cells
7. Dependent upon intact bursa or supply of B cells
8. Two or more antigens per germinal centre
9. Antigens localised in form of antigen-antibody complexes through Fc portion of antibody molecule
10. B cell homing site
11. Once in germinal centre B cells undergo rapid division presumably under influence of antigen
12. B cells in intimate contact with antigen on dendritic cells
13. Interaction site between T cells, B cells, and macrophages
14. B cells are immunocompetent because occasional specific antibody-producing cells are seen in germinal centres
15. At the peak of germinal centre formation antibody production drops sharply
16. At the peak of germinal centre formation no antigen-specific plaque-forming cells are found in the circulation

How may these numerous observations be woven into a common mechanism of action? The following sequence of events is proposed to explain the action of Freund's adjuvants on the antibody response of the chicken. It is based both upon White's hypothesis of 1973 and the work of Sercatz and Coons in 1962. These latter authors proposed that three populations of antigen sensitive cells were important during an immune response, (and one must remember that this was in the days before T and B sub-populations), and called these the X, Y, and Z cells. The X cells were virgin cells, previously unexposed to antigen, the Y cells were memory cells (B memory cells) and progeny of X cells after exposure to antigen, and Z cells were fully differentiated antibody-producing or plasma cells. The X cells could differentiate to either Y or Z cells. These authors found, during attempts to transfer secondary immune responses in rabbits, that there was a significant lag phase after a primary response before transfer of a spleen cell suspension of an immunised animal into an unimmunised recipient would also transfer a secondary response. This lag phase was thought to be the time required for the full expansion of X cells into Y cells.

In the present model the initial event, of course, is the intramuscular injection of FCA or FIA plus a protein antigen. Almost immediately some of the oily antigen-adjuvant mixture escapes into the circulation via the lymphatics. There follows an immune response which involves two different tissue sites, the spleen and the local granuloma. This scheme of events is shown diagrammatically in Fig. D.1.

In the spleen plasma cells forming antibody against the protein antigen (HSA) appear and within 72 hours of injection circulating antigen, in the form of antigen-antibody complexes, begins to localise to the ellipsoids or Schweigger-Seidel sheaths. These complexes are taken

Fig. D.1. Diagrammatic representation of possible mode of action of Freund's adjuvants on antibody biosynthesis.



(●) Germinal centre

(1) Initial intramuscular injection

(2) Antigen localisation to spleen

(3) Germinal centre formation in the spleen and FIA granuloma

(4) Release of B memory cells from the spleen

(5) Trapping of B memory cells in germinal centres of granuloma (FIA only) or transformation to antibody-producing cells.

(6) Antibody production

up either by macrophages or by dendritic cells. The dendritic cells then migrate through the white pulp to specific sites in the organ where they form clusters with B cells which eventually become recognisable as germinal centres.

At roughly the same time the tissues at the site of injection are undergoing a similar process, the only difference being that in this case the antigen does not localise to the lymphoid tissues, the lymphoid tissues localise to the antigen. There is a local invasion of a number of different cell types, polymorphonuclear cells, monocytes, macrophages, lymphocytes, plasmablasts, and dendritic cells, in fact all the cells necessary to form regular lymphoid tissue. These cells rapidly colonise the lesion and form a splenic analogue so that there soon takes place an identical process of antigen-dendritic cell uptake and germinal centre formation (process 3 in Fig. D.1.).

The formation of germinal centres in the spleens and granulomata of the FIA birds signals the cessation of antibody production in both sites as potentially antigen-reactive B cells are sequestered in the centres under the influence of the antigen on the surface of the dendritic cells, and presumably in a state of tolerance.

In the FCA birds however, a similar process of germinal centre formation in the granulomata is interfered with by the presence of the mycobacteria or their components so that at one week only a few badly distorted centres are visible, and after this time none at all. This event effectively removes the major homeostatic control mechanism relating to antibody biosynthesis from the granulomata.

From the 15th day after injection onwards the germinal centres in the spleens of both FIA and FCA groups of birds begin to release their (by now) vastly expanded number of B memory cells back into the

circulation - the sequestration of these cells probably corresponds to the lag phase of Sercatz and Coons, and the release of these B memory cells to the appearance of large numbers of Y cells and the potential to transfer a secondary response. This is process 4 on Fig.D.1. These memory cells circulate through the blood and many exit through reactive blood vessels in the granulomata.

In the FCA animals the antigen-specific B memory cells encounter abundant local deposits of antigen, many T cells, and numerous macrophages, a combination which is particularly conducive to antibody formation (Feldmann, 1972). This results in a spectacular burst of antibody (6-Fig.D.1) production as each specific B memory cell is triggered off by local antigen to become a plasma cell (Z cell).This can often be seen on histological sections of FCA granulomata, in which small mononuclear cells are visible in and immediately surrounding small vessels. Slightly further into the tissue are cells with a plasmablast morphology and further still are mature plasma cells, many of which can be shown by immunofluorescence to be making specific antibody. This mechanism would explain the extremely high levels of antibody measured in these birds. Even four weeks after injection some splenic germinal centres containing the protein antigen are still visible so presumably there is still seeding of B memory cells to the granulomata. Once in the granulomata the plasma (Z) cells probably continue antibody production until they exhaust their capacity.

In the FIA birds however, any B memory cells which migrate from the spleen to the granulomata come under the homeostatic influence of the many local germinal centres and are probably re-sequestered in these structures. Obviously some of the memory cells are converted to plasma cells or Z cells because there is histological evidence of minor antibody production in the lesions, as well as an occasional rebound seen in the circulating antibody responses of these animals.

Similar mechanisms in other depot-type adjuvants

If such a feedback mechanism is operative in the chicken, and is mediated through germinal centre formation, then perhaps further evidence would be available from a study of other depot-type adjuvants. For example, the mode of action of alum salts and water-in-oil emulsions is considered to be similar, in that they both act to form a depot from which antigen is slowly released (White, 1967a). In the alum depot however, antigen, despite its continued presence, rapidly fails to act as a stimulus for antibody production so that at one month after injection the serum antibody level of recipients is already markedly decreased. It was thought that this was due to a process of fibrous encapsulation of the depot site, effectively preventing further release of antigen (White, 1967b).

Some preliminary observations on the effect of alum on the chicken (White and Smithyman, unpublished results) are therefore of relevance here. Injection of HSA in alum into the breast muscle of a chicken, produced, on examination four weeks later, a small bleb of tissue between the muscle fibres, which proved to be the alum granuloma. In addition to large numbers of lymphocytes and alum-containing macrophages the granuloma contained several extremely large germinal centres, one of which is shown in Figure D 2. These germinal centres could be shown to have the normal dendritic cell surface pattern of antigen, in this case HSA. There were few anti-HSA antibody-producing cells visible. It would seem that a similar germinal centre-mediated feedback system to that seen with water-in-oil emulsions (FIA) is operating in this situation.

Fig. D 2. Alum granuloma in the breast muscle of a chicken.

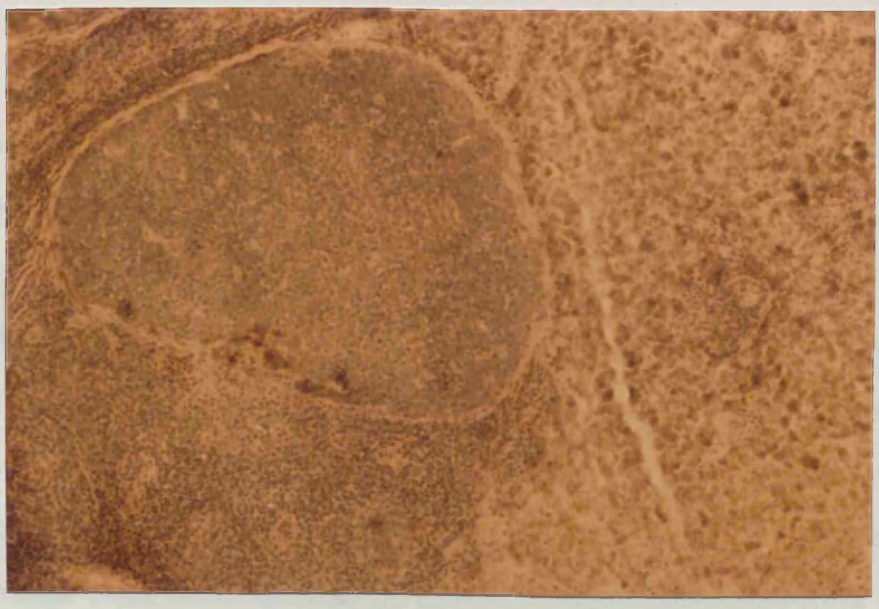


Fig. D 2. Photomicrograph of a section of an alum granuloma from the breast muscle of a chicken injected 4 weeks previously with 1mg of HSA in 0.5 ml of alum. To the right of the picture is a continuous sheet of alum-bearing macrophages. The left half of the picture is occupied by a dense lymphoid area, the major portion of which is given over to an enormous germinal centre.

Dominici stain x 180

Comparative findings in other studies and in other species

The chicken is obviously a special case as it has no lymph nodes in the conventional sense, but is there any evidence of disruption by Freund's adjuvants of the lymphoid tissues of other species and if so can this model explain some of the effects of Freund's adjuvants on these species as well?

Though mostly circumstantial and usually confined to histological descriptions a number of observations in previous studies suggests that such evidence is available.

Fischel and his colleagues (1952) examined the role of FCA in production of antibody to egg albumin by guinea pigs. They provided elegant photographic evidence of " an unusual histological finding ... .which does not appear to have been previously described.....In the majority of these spleens the Malphigian corpuscles were surrounded by a rather heavy rim of fibrous tissue arranged in concentric lamellae.....".

Askonas and White (1955) observed that, in guinea pigs injected in the footpad with FCA, the normal architecture of the draining lymph nodes (popliteal, lumbar, and flank) was almost totally replaced by granulomatous tissue. Presumably germinal centre development was also affected.

In contrast to this Gaafar and Turk (1970), in a study of granuloma formation in guinea pig lymph nodes showed that though FIA resulted in considerable disruption of tissue structure ".... the lymph node germinal centres appeared untouched and normal in appearance.".

Moore and his colleagues (1963) followed the effects of an intravenous injection of FCA and diptheria toxoid on the spleen and lymph nodes of rabbits. While observing no effects on germinal centre histology they concluded that " ...initially the cellular reaction corresponds to that of a primary response to a bland antigen, but the changes are more florid. This is followed by an uninterrupted progression to the type of cellular changes associated with a secondary response, instead of the usual sequence of a primary response in which the tissues begin to return to normal by 6-8 days. ". This suggests that here too normal

homeostatic mechanisms have been affected by the adjuvant.

Good (personal communication) has stated that in studies carried out in his laboratory it was a common finding that the homolateral lymph nodes of FCA treated mice were lacking in germinal centres while the contralateral nodes contained normal numbers.

Humphrey has mentioned the disrupting effect of FCA on germinal centres on a number of occasions (Humphrey, 1975; Humphrey et al, 1972; Balfour and Humphrey, 1967). For example, in a study of antigen localisation to lymph node germinal centres of mice primed with antigen in FCA (Balfour and Humphrey, 1967) the authors commented " In these experiments Freund's adjuvant had to be used in the primary immunisation and this resulted in great distortion of the lymph node architecture, in particular of the germinal centres, which were often difficult to recognise as such. ".

Finally White (1973) described an experiment in which FCA was administered intravenously to chickens (i.e. in the same species) as a water-in-oil-in-water (WOW) emulsion (Herbert, 1973). One striking effect of this adjuvant emulsion was the appearance of germinal centres in the spleens of the recipient animals which were far smaller than in controls and in addition were often badly distorted, with occasional small epithelioid granulomas at their periphery.

The nature of the interference mechanism

The exact nature of the mechanism of interference of germinal centre formation by mycobacteria in FCA is unknown. Three possible mechanisms are suggested here:-

a) a physical displacement effect

b) an allergic or delayed hypersensitivity effect

c) a specific physiological effect at the cell surface level

a) physical displacement effect

It has been noted by Turk (1973) that in patients and animals suffering from chronic infections such as leprosy, congenital syphilis, sarcoidosis, and malaria the thymus-dependent areas of the lymph nodes and spleens may be depleted of T cells and replaced with another cell population,usually macrophages in the case of the first three diseases, or plasma cells in the case of malaria. If, as has been suggested by Thorbecke and Lerman (1976), T and B cell interaction is required for germinal centre formation, then replacement of the T-cell areas would have an adverse effect.

b) allergic or delayed hypersensitivity effect

White (1973) found that, in the spleens of chickens given mycobacteria in a water-in-oil-in-water emulsion, small germinal centres were visible with small granulomatous reactions at their periphery. Similar centres were seen in this study in local granulomata. It is possible that the granulomatous reactions are due to an allergic or delayed hypersensitivity response to the mycobacteria which have been carried to these sites by macrophages. If so then this response could be sufficient at a purely local level to interfere with the formation of germinal centres.

c) specific physiological effect at the cell surface level

Far more appealing than the above possibilities is an interference mechanism which is specific to cell surfaces. The work of Feldmann (1972a and b) and Feldmann and Basten (1972) suggests a suitable cell surface - that of the macrophage. These authors were able to develop an elegant *in vitro* model of antibody synthesis based on a double chamber culture

flask, separated by a cell-impermeable membrane. In the top chamber were antigen-activated T cells and in the bottom macrophages and primed or unprimed spleen cells. These macrophages proved capable of initiating an immune response from the spleen cells, after activation by a factor diffusing across the membrane, which later proved to be a complex of antigen and monomeric IgM antibody. Removal of the macrophages from the system prevented the immune response. Feldmann proposed that complexes of antigen and T cell antibody formed a lattice on the surface of the macrophages which stimulated potential antibody-forming B cells.

In the case of germinal centre formation in the chicken a similar interaction may occur, between T cells, B cells, and dendritic cells. Though it is not yet clear whether dendritic cells are simply specialised macrophages or not, they also carry a matrix of antigen and antibody on the cell surface, and interact with B cells to form germinal centres (White et al, 1970). If the antigen-antibody complexes are prevented from loading onto the dendritic cell surface then germinal centre formation will be effectivly inhibited.

It is of interest therefore to consider the following finding. Stewart-Tull and Wilkinson in 1973 showed that mycobacterial glycopeptides have an affinity for guinea pig γ2-immunoglobulin, which is cytophilic for homologous macrophages. The glycopeptide did not interfere with the antigen-reactive sites on the antibody molecule. Mycobacteria then, may produce their effect by preventing or altering the normal cytophilic attachment of antigen-antibody complexes to the surface of the dendritic cell, thus preventing germinal centre formation and consequently antibody homeostasis.

Other Phenomena Associated With the Use of Freund's Adjuvants

Let us consider the feedback model in the light of other well known phenomena associated with the use of Freund's adjuvants.

a) High immunoglobulin levels

On the basis of the model proposed here is it possible to explain the finding of high 7S levels found after use of FCA? Humphrey (1963) found that FCA injected intramuscularly in rabbits produced a considerable rise in the level of 7S-immunoglobulin, only part of which he could absorb out with mycobacterial antigens. When a protein antigen (HGG) was added to the FCA a major proportion of immunoglobulin was composed of antibody to the included antigen. The suggestion has been made that a proportion of the immunoglobulin was directed against new antigenic components caused by a denaturation effect of the oil on protein. This has however been disproved by two recent studies on the effect of FCA on haemoglobin (Berzofsky et al., 1977) and on myoglobin (Smith et al., 1977). Both groups demonstrated little or no denaturation of the protein by the FCA even after prolonged storage.

More recently Humphrey and Monteiro (personal communication - 1975) described an experiment in which they followed immunoglobulin and antibody levels in various groups of mice injected i.p. with FCA / DNP-BSA. The groups included thymectomised, irradiated and bone marrow reconstituted (T x B), irradiated and bone marrow reconstituted (x B), and unoperated CBA x C57 FI mice and also nude (nunu) mice. The T x B mice unexpectedly showed markedly greater increases

in non-specific immunoglobulin levels than other groups, though they made no antibody. Such increases were not observed in the nude mice. The authors proposed that the increases were due to a B cell stimulatory factor released by activated macrophages and that the TXB mice responded most because they lacked inhibitory T cells. Nude mice are totally unable to form germinal centres while TXB mice, though deficient in this respect, still can (see page 144). This probably indicates that a number of T cells are still present in the TXB animals.

These germinal centres still present in the TXB mice probably contain antigens to which the animals are daily exposed. Freund's adjuvant, by interfering with these centres destroys an already crippled homeostatic mechanism, with a consequent production of antibody to all the environmental antigens concerned. The proportion of immunoglobulin synthesised against each antigen probably depends on the proportion of the germinal centres occupied by that particular antigen. For instance, in the first experiment above, the major part of the immunoglobulin when no protein antigen was included was directed against the mycobacterial antigens. When a protein antigen was included a large part of the resulting immunoglobulin was directed against it.

These interpretations are not necessarily incompatible as the mechanism by which FCA interferes with germinal centres could involve the co-operation between T and B cells thought to take place at the edge of the germinal centre.

b) $\gamma 1, \gamma 2$ immunoglobulins

A well known, but as yet unexplained effect of mycobacterial adjuvants, is their effect on the class of antibody produced. In

the guinea pig for example, a protein antigen, which is injected alone or in a water-in-oil (FCA) emulsion, leads to an antibody response at three weeks which is almost entirely confined to the fast or $\gamma 1$ globulin fraction (as determined electrophoretically). When mycobacteria are added to the water-in-oil emulsion and antigen, a new peak of slow or $\gamma 2$ immunoglobulin appears (White, Jenkins and Wilkinson, 1963). The two classes may be readily separated chromatographically, they have a similar light chains but different heavy chains, and have different physiological properties. For example $\gamma 2$-immunoglobulin fixes complement whereas $\gamma 1$ does not, they have different Fc pieces, and $\gamma 2$ possesses cytophilic activity for homologous macrophages, while $\gamma 1$ does not (White, 1976), and they are produced in different plasma cells (Nussenzweig and Benacerraf, 1967, 1968).

In the model proposed above the rise of $\gamma 2$ globulin may be explained on the basis of interference of the feedback mechanism, if the $\gamma 2$-immunoglobulin is normally active in homeostasis of antibody production by virtue of its attachment to dendritic cells, and participation in germinal centre formation, but at levels not normally detectable by immunoprecipitation techniques. If the attachment of $\gamma 2$ is prevented by mycobacteria then increased production and accumulation of the immunoglobulin, to detectable levels, may be induced to counteract this.

c) Induction of auto-immune disease by FCA.

One of the most successful uses of FCA has been in the production of experimental auto-immune diseases such as allergic encaphalomyelitis, thyroiditis and aspermatogenesis. Though obviously

successful, research workers have been somewhat at a loss to explain this induction of auto-immunity. It seems clear that the natural tolerance of the body to its own constituents is broken in some way by the mycobacteria, but how this is brought about is obscure. It is proposed (Weigle, 1971) that auto-antigens (such as thyroglobulin) circulate in low concentration in the body fluids under a state of tolerance maintained by T cells. This low dose tolerance state is obviously broken by the presence of auto-antigen in FCA (White, 1976). In the model proposed here antibody production and germinal centre formation would take place due to the presence of abnormal amounts of auto-antigen. This would lead to an expansion of both T and B memory cells which, when released back into the circulation, are driven by antigen emerging from the depot site to respond with a cell-mediated and humoral immune response against organ and tissue sites.

The antigen is obviously presented to the immune system in a significantly different way, perhaps in a higher antigen/antibody ratio than normal which results in a slight immune response against that antigen. In the normal course of events this would be quickly suppressed by feedback mechanisms operating through germinal centre formation but in this case the expanded immuno-competent memory cells reach the injection site or local lymph nodes, and under the influence of high, local concentrations of antigen and lacking the homeostatic control of germinal centre formation a vigorous cell mediated or humoral immune response or both takes place, possibly with the generation of high antibody levels. These changes will then lead to damage at the organ sites involved.

Suggestions for future research

The experiments described here inevitably suggest further experiments to be carried out. Some of these are given below:-

a) Proof of seeding of lymphocytes from spleen to granuloma.

One of the main tenets of the hypothesis advanced here is that the spleen acts as a trainer and supplier of cells to the granuloma. In this study it was difficult to establish this as inbred chickens were not available. If they were it would be relatively simple to give birds an antigen in FCA or FIA, kill them at various time intervals after injection, make a white blood cell suspension from the spleen, label this and inject it into syngeneic recipients bearing FCA and FIA granulomata of various ages. The prediction would be that large numbers of immunocompetent cells would home to the granuloma from the second week onwards.

b) Cell populations in the granuloma.

Methods are now widely available for the identification of different lymphoid cell populations by surface markers, rosetting (E, EA, and EAC), and enzymatic techniques. It would be of great value to characterise the different cells which colonise granulomas on this basis as this may suggest new approaches to the treatment of granulomatous disease.

c) Mechanism of action of Freund's adjuvants.

It should be possible to perfect a simple cytophilic antibody test, using antigen-coated red blood cells, cytophilic antibody, and macrophages (Rosa Vasquez - personal communication). The level of cytophilic antibody would be reflected in the number of rosettes formed between macrophages and red blood cells. A suitable source of macro-

phages is the peritoneum of the chicken. Once established the cytophilic antibody test could be performed in the presence of mycobacteria, mycobacterial products, or other adjuvants to see if there is interference with the attachment of the antibody to the macrophage surface.

d) Germinal centre separation.

Using the technique of germinal centre separation described in the results (Exp. 6.6.) there are a number of important experiments which should be attempted.

1) It may be possible to obtain pure suspensions of germinal centre cells to discover their ultimate destination (through labelling studies) and whether they can transfer immunological memory from one animal to another.
2) The germinal centre cells could be cultured.
3) The germinal centre itself could be cultured.
4) It would be of interest to see under what conditions one could convert an isolated germinal centre from a primed animal to an " antibody " centre, for instance by adding the priming antigen to the culture medium.
5) Could one create an artificial germinal centre by adding together the correct numbers of T cells, B cells, antigen-bearing dendritic cells, and a capsule?

Relevance of this study to current immunological problems

The experiments carried out in this study, though restricted to the avian species, may be relevant to immunological problems affecting man as well. These problems include granulomatous disease, germinal centre function and mode of action of immunotherapeutic adjuvants.

a) Granulomatous diseases

A glance at the list of diseases which involve granuloma formation (page 26) gives a rough idea of the extent of this problem. After more than a century of investigation the aetiology of many of these diseases is only poorly understood or not at all. It is to be hoped that a logical approach to the study of the cells which characterise granulomatous lesions, especially in the light of recent advances in immunobiology, may lead to a greater understanding of the underlying causes and to a more effective therapy. In this respect an appreciation of the role of the lymphoid tissue component of the granuloma, such as in this study, may be of value.

b) Germinal centre function

Germinal centres are an important component in the immune response of many species. They are involved in that most crucial of immunological phenomena, memory. They also provide a site for long-term antigen retention, B cell homing, T and B cell and macrophage interaction, for immunological tolerance, and perhaps, as reported in this thesis, for homeostasis of antibody production.

The immune system, far from being a simple mechanism, is gradually being revealed as an incredibly complex network of interacting cells and cell products, which act to enhance or suppress or modify the different effector arms of the immune response. Germinal centres, by virtue of their involvement in the many aspects of the immune response listed above, are ideally suited to play a central role in immune enhancement or suppression. In other words the germinal centre may act as the fine tuner and amplifier of the immune response. It is hoped that the imformation regarding the kinetics of antigen and bursa cell localisation to

germinal centres reported here, and the technique developed for the separation of whole germinal centres in vitro, may prove of great value in elucidating these control mechanisms.

c) Immunological adjuvants

A guiding factor behind immunological research is the hope that, at some point in the future, the workings of the immune system will have been unravelled to such an extent that we may be able to manipulate it at will, both for preventative and therapeutic purposes. At the present time there has been assembled, on a purely empirical basis, a large and varied group of compounds, the immunological adjuvants, which are known to modify the immunological response in some way. Research in this field is now being directed to developing adjuvants specific for individual cell types. For example, levamisole, BCG, and C.parvum affect cell-mediated immunity while lipopolysaccheride (LPS) and various polyanions affect humoral immunity.

Perhaps the most important application of adjuvants at the present time is in the immunotherapy of cancer. Despite the success of some of the animal experiments (for example Zbar et al., 1971) and of some clinical studies (for example Mckneally et al., 1977) the results to date have not been encouraging. This has led to a rather depressing hiatus, as summed up recently in an address given by Dr. Terry of the U.S National Cancer Institute(personal communication, 1977). In referring to the many controversial and often disappointing results of cancer immunotherapy trials he concluded that " There are no clinical studies available which make immunotherapy a standard treatment for cancer in human patients. Perhaps in one to two years there will be more definite trends. The hope

for immunotherapy lies with the rapid advance in immunobiology and the rapid dissection of the immune response. Until the immunologist provides the clinician with better tools and a better understanding of the interaction between immunology and cancer he (the clinician) can only wait.".

It is hoped that this study on the modulation of the immune response by Freund's adjuvants will have helped in some way to achieve such an understanding.

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