SITES OF ANTIBODY PRODUCTION IN THE GUINEA-PIG. THE RELATION BETWEEN *IN VITRO* SYNTHESIS OF ANTI-OVALBUMIN AND γ-GLOBULIN AND DISTRIBUTION OF ANTIBODY-CONTAINING PLASMA CELLS

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MANY authors maintain that the members of a cytological family whose mature member is the plasma cell are responsible for formation of antibody globulin (Bjørneboe and Gormsen, 1943; Fagraeus, 1948; Marshall and White, 1950; Ringertz and Adamson, 1950; Keuning and van der Slikke, 1950; Coons, Leduc and Connolly, 1955). When an antigenic stimulus is administered intravenously the multiplication and differentiation of these cells has been found to occur mainly in the spleen, liver and lung. When the antigen is distributed by lymphatics from a subcutaneous focus such cells appear in the regional lymph nodes. In the latter instance, and especially when the antigen is mixed with " adjuvants ", local granuloma formation takes place at the site of injection. Under certain circumstances plasma cells compose a fair proportion of the cells of such granulomata. When the adjuvant is alum or aluminium phosphate considerable numbers of plasma cells are present at the site of injection and such cells have been regarded as a source of antibody (White, Coons and Connolly, 1955). On the other hand, local formation of antibody in granulomata produced by alum adjuvant has been regarded by Hartley (1940) as evidence that the macrophages function in this respect. Similarly, the local cellular response following the use of Freund-type adjuvants (Freund and McDermott, 1942), in which the antigen is administered in a water-in-oil emulsion with added *Mycobacterium tuberculosis*, has given rise to the view that the cells assembled near the site of injection are responsible for antibody production (Freund, 1951). The main cell component of such granulomata is the macrophage.

Analysis of such problems has been attempted by the extraction of antibody from the local site at varying time intervals after injection of antigen. Westwater (1940*b*) showed by such studies that complement-fixing antibody to tubercle bacilli appeared earlier in the lesion at the site of injection than in the blood. This method only proves *localisation* of antibody; that *production* of antibody occurs at the site of its localisation requires further support.

Further evidence for determining the source of such antibody was provided by the method for the cytological localisation of antibody (Coons *et al.*, 1955). Applied to the guinea-pig (White *et al.*, 1955), this method showed that the macrophages of the local granuloma were devoid of antibody. On the other hand, large numbers of antibody-containing plasma cells were present in the lymph nodes remote from the site of injection, and in the spleen.

The present investigation was undertaken to correlate these findings with the ability of the isolated tissues to synthesise specific antibody when incubated *in vitro*, using the method of Askonas and Humphrey (1955). Data are also provided concerning the synthesis of γ-globulin under the same experimental conditions. Preliminary findings of this study have been reported (Askonas and White, 1955).

MATERIALS AND METHODS

Antigen and mode of administration

The antigen used throughout this study was crystalline egg albumin (Armour and Co., Ltd.) in doses of 5 mg. per injection. This was given in 0·2 ml. of a water-in-oil emulsion into the subcutaneous tissue of the footpad. This site was chosen so that draining lymph nodes in the popliteal fossa and those of the flank could be conveniently used. The injection mixture was composed of 1 vol. of a saline solution of antigen, 1 vol. of Arlacel A (batch 3375, Atlas Powder Company, Wilmington, Delaware) and 3 vol. Bayol F (Esso Standard Oil Company). Tubercle bacillary wax (lipopolysaccharide or wax D fraction of the human strain " Canetti "), kindly supplied by Dr. E. Lederer of the Institut de Biologie Physico-chimique, Paris, was dissolved in the Bayol F before emulsification to give a dose per injection of 1 mg.

Animals

Guinea-pigs of weight 350–500 g. were used. They were fed on a pellet diet with supplementary cabbage *ad lib.*

Preparation of tissues

Animals were killed 3 weeks after injection by inhalation of chloroform. Immediately after death the blood was removed by heart puncture. The granuloma in the injected footpad was dissected free from the underlying metatarsal bones and the overlying skin. Samples from this and other organs were immediately weighed, and preserved for histological study by placing against the wall of thin glass test-tubes and quickly freezing in alcohol-CO_2 mixtures at $-$ 70°. They were stored at $-$ 20° until frozen sections could be prepared.

The remainder of the tissues was sliced with an automatic chopper (McIlwain and Buddle, 1953) and with scissors by hand and then incubated to estimate their ability to synthesise antibody *in vitro*. Known weights of sliced tissue (0·1–1·0 g. depending on the level of activity) were incubated in siliconed flasks at 37° in an isotonic salt mixture at pH 7·4 (Raker, Taylor, Weller and Hastings, 1950) containing 0·3 per cent glucose in a total vol. of 4 ml. Each flask contained 1·7 mg. of a complete amino acid mixture (acid-hydrolysed rabbit plasma protein plus tryptophan), 1 ml. of the animal's own serum and 2–3 μc of uniformly labelled ^{14}C-glycine (0·05 μc per μg.) Carbon dioxide (5 per cent) in oxygen was passed continuously through the flask and mechanical shaking was maintained throughout.

The reaction was stopped after 3–4 hr. by adding sodium ethylmercury-thiosalicylate (Merthiolate : Eli Lilly and Co.) to a final concentration of 1/10,000 ; antiserum was added so that each flask contained at least 5 mg. of antibody, and in order to dilute the radioactivity of the glycine in the system, inactive glycine was added to produce a 1/1000 dilution. The incubation mixture was frozen by immersing the flasks in a solid CO_2-ethanol mixture. After thawing the cells were broken in a glass homogeniser of the Potter-Elvehjem type and the cellular particles centrifuged down at 18,000 g. at 1°. The supernatant contained the anti-ovalbumin and γ-globulin fractions.

Isolation of specific antibody precipitate

Antibody was precipitated from the supernatant by addition of the calculated amount of ovalbumin, assuming an optimal precipitating ratio for ovalbumin to anti-ovalbumin of approximately 1 : 10. This ratio had been determined by the construction of precipitation curves relating weight of precipitates to antigen concentration for several antisera from guinea-pigs immunised with ovalbumin in adjuvant mixtures similar to those used in these experiments. Precipitation was allowed to continue during 15 min. at 37° and overnight at 0–4°. Precipitates were washed three times with 1 per cent glycine-saline, once with distilled water, once with hot ethanol and dried with ether.

Preparation of γ-globulin fraction

After removal of the specific antibody precipitate, a γ-globulin fraction was prepared by the method of Kekwick (1940) for human γ-globulin. The protein was precipitated first with 18 per cent w/v of sodium sulphate. It was then reprecipitated twice with 15 and 12 per cent of sodium sulphate. A small volume of saline was added and the protein soluble in saline was dialysed against distilled water and freeze-dried. This represented the γ-globulin fraction. This fraction is not homogeneous in guinea-pigs, but contains mostly the components of serum which move slowest during electrophoresis at pH 8·4 in veronal buffer, $I = 0{\cdot}2$.

Determinations of radioactivity

Dried protein was plated at infinite thickness on 0·3 or 1 sq. cm. disks and radioactivity determined by counting in an automatic recording Geiger counter with a thin-end window to an accuracy of at least ± 5 per cent.

Morphological techniques

Frozen sections at 4μ thickness were prepared from the unfixed tissue samples by the techniques of Linderstrøm-Lang and Mogensen (1938) as modified by Coons, Leduc and Kaplan (1951). The fixation and histochemical demonstration of anti-ovalbumin followed the method of Coons, Leduc and Connolly (1953, 1955) as applied to this antibody (White *et al.*, 1955). Fluorescence microscopy was carried out using equipment as described by Coons, Creech, Jones and Berliner (1942) including the modification of a dark field condenser (Zeiss cardioid) (Coffin, Coons and Cabasso, 1953). Anti-ovalbumin was seen in sections viewed with the fluorescence microscope as bright apple-green areas. A control section for the detection of ovalbumin antigen, which will also reveal any non-specific staining due to the use of the fluorescein-antibody conjugate, was always set up at the same time as that for the detection of specific antibody. A description of this control and of such non-specific staining in the lymphoid tissues of the guinea-pig is given in White *et al.*, 1955.

Counts of antibody-containing cells in tissue sections

Sections stained for anti-ovalbumin were scanned under the fluorescence microscope using a 3·65 mm. oil-immersion objective and a × 4 ocular, into which was incorporated a divided square field of 6 mm. side. The total area of section seen within the boundaries of this square was 0·0324 sq. mm. The cells within an area of 30–60 squares were counted and results were expressed as counts per unit square. Since the distribution of such antibody-containing cells is not uniform throughout a lymph node, such counts only gave a very rough indication of relative cell densities. Moreover, counts made in that portion of a bisected node which is sectioned and studied morphologically can give only a vague idea of the numbers of cells in the other portion which was incubated *in vitro*.

RESULTS

Histological Findings

All animals were killed 21 days after the single injection of ovalbumin-adjuvant mixture into the left foot-pad. The following is a simplified summary of the histological findings at autopsy. For fuller details reference should be made to two publications (White *et al.*, 1955; Suter and White, 1954) dealing with identical findings with similar adjuvant mixtures. The cytological terminology has been defined previously (Marshall and White, 1950).

Granuloma at site of injection.—The foot was greatly swollen due to the presence of firm, yellowish-grey tissue, mainly in the plantar region. This lesion was a mixed-cell granuloma. The predominating cells were macrophages, often modified to epithelioid cells and giant cells, forming a solid proliferation around oil vacuoles. Lesser numbers of polymorphs and eosinophiles were present. Plasma cells were rare.

Sections which were treated to reveal antibody to ovalbumin showed that the main mass of cells—the macrophage elements—were devoid of fluorescence. Antibody-containing cells were very scanty and limited to groups of 2–3 cells in the adventitia of small vessels (Plate I). Adjacent sections stained with Giemsa revealed these as mature and immature plasma cells. The presence of large amounts of extracellular antibody was indicated by brilliant fluorescence of the surface of collagen fibres in the area.

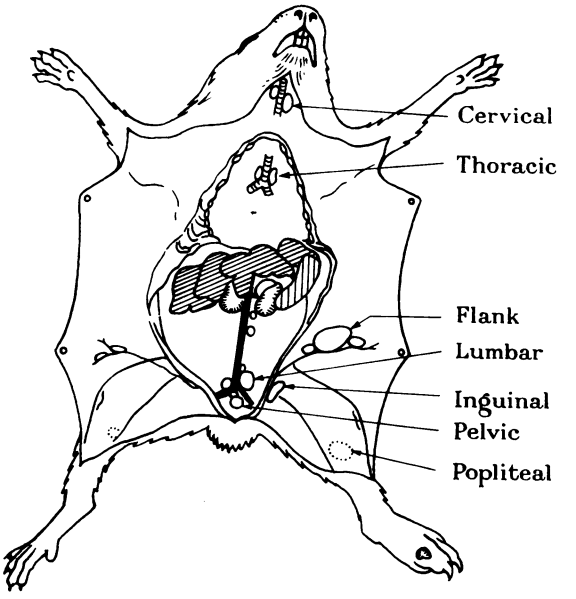


FIG. 1.—Drawing of a guinea-pig injected with the ovalbumin-adjuvant mixture in the left foot-pad, showing the position of the lymph nodes which were studied.

Lymph nodes.—The regional lymph nodes of the homolateral popliteal fossa were enlarged to 3–5 times normal mass (Fig. 1). Their normal structure was almost totally replaced by granulomatous tissue consisting of macrophages. Sections treated to demonstrate the presence of antibody showed a few antibody-containing

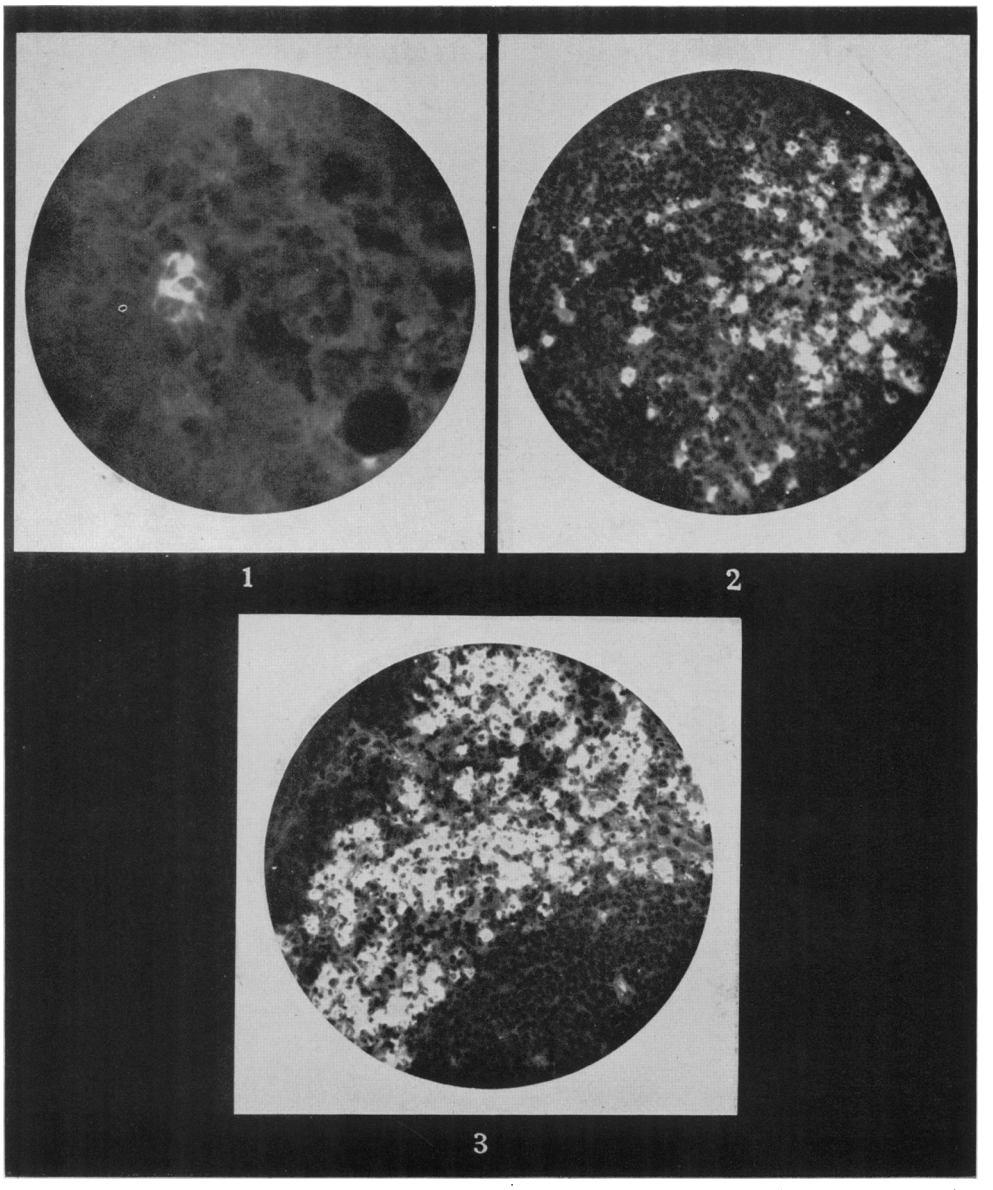
EXPLANATION OF PLATES

Plates I–III are fluorescence micrographs of frozen sections of guinea-pig tissues which were treated to reveal the distribution of anti-ovalbumin. The light areas represent the deposited fluorescein-labelled anti-ovalbumin which appeared bright apple-green in the original preparations. The primary blue fluorescence of the tissues gives a dim outline of other structures.

PLATE I.—Three antibody-containing plasma cells in the granuloma at the site of injection of ovalbumin-adjuvant mixture in the foot-pad of the guinea pig. The antibody outlines the cytoplasm of these cells. ×840.

PLATE II.—Homolateral flank lymph node. The bright cytoplasmic outlines of antibody-containing plasma cells are seen scattered over the section. × 190.

PLATE III.—Contralateral lymph node. Dense collections of antibody-containing plasma cells throughout a medullary cord between the edges of two lymphoid nodules (above left and below right) which are devoid of fluorescence. × 190.



Askonas and White.

cells, identifiable as immature or mature plasma cells by reference to adjacent Giemsa-stained sections.

Strikingly enlarged to 8–12 times normal mass were the lymph nodes of the homolateral flank (forming a group of 2–4 nodes along a vein which descended from the side of the abdomen to the inguinal region). Sections showed large islands of granulomatous tissue made up mainly of masses of macrophages, with strands of lymphoid tissue between these and at the periphery. These strands contained many plasma cells. Appropriately treated sections showed the presence of many antibody-containing cells (identifiable as of the plasma cell series) within such lymphoid strands (Plate II).

The lumbar nodes were also many times enlarged and largely occupied by macrophage granulomata. Some plasma cells were present.

Other lymph nodes which were more remote from the injected foot-pad had much less macrophage tissue. The nodes of the contralateral flank region were only slightly enlarged and macrophage masses were absent. The main feature here was an intense plasma cell infiltration in the medullary strands, and tissue sections treated to demonstrate antibody showed large numbers of antibody-containing cells in this situation (Plate III).

Other lymph nodes remote from the site of injection, such as the thoracic and the cervical, also had many antibody-containing cells in colonies around the medullary vessels.

Liver.—The only histological change was the presence of small collections of lymphocytes, with scanty plasma cells, in the portal spaces. In appropriately stained frozen sections an occasional antibody-containing cell could be found in such areas.

Lungs.—Numerous sub-miliary granulomata were present, mainly subpleurally, and composed principally of macrophages with epithelioid characteristics and giant cells. Eosinophiles were also present; there were few lymphocytes and plasma cells. Appropriately treated sections revealed a few antibody-containing plasma cells.

Spleen.—Groups of macrophages were present in the medulla and within the sinuses. Colonies of antibody-containing cells identifiable as plasma cells were found grouped around medullary vessels.

Table I gives in simplified form the approximate relative densities of macrophages and antibody-containing plasma cells at various sites of the injected guinea-pig.

TABLE I.—*Approximate Density of Macrophages and Antibody-containing Plasma Cells in Different Tissues of the Immunised Guinea-pig*

	Macrophages.	Anti-ovalbumin-containing plasma cells.
Local granuloma of injected foot	+++	±
Popliteal lymph node	+++	±
Homolateral flank lymph node	++	++
Lumbar lymph node	++	±
Contralateral flank lymph node	±	+++
Cervical lymph node	±	++
Lung granulomata	+++	±
Liver	±	±
Spleen	+	+

Incorporation of Radioactive Amino-acids into Antibody and γ-Globulin Fraction 'in Vitro'

Fig. 2 records the incorporation of ^{14}C-glycine into the anti-ovalbumin and γ-globulin fractions at suitable time intervals by lymph nodes from the flank of immunised guinea-pigs. It can be seen that after an initial lag period the rates of

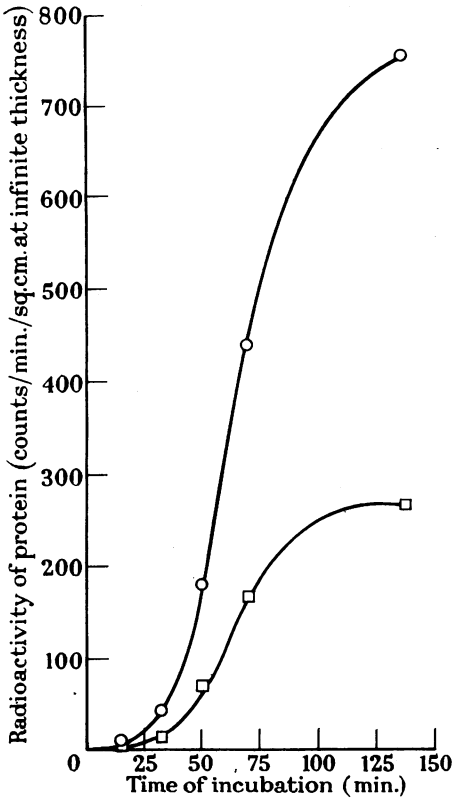


FIG. 2.—Time curve showing the incorporation of ^{14}C-glycine into anti-ovalbumin and γ-globulin fractions. ⊙ anti-ovalbumin; □ γ-globulin fraction. Sliced lymph nodes from the homolateral and contralateral flank of immunised guinea-pigs were incubated at 37° in an isotonic salt medium containing 0·3 per cent glucose, 2·5 μc ^{14}C-glycine and 1·7 mg. of an amino acid mixture; the gas phase was 5 per cent CO_2/95 per cent O_2.

incorporation of both anti-ovalbumin and γ-globulin fractions remain steady over a 2-hr. period, and then rapidly fall off. The term "γ-globulin fraction" refers to the salt-precipitated protein (*vide* Materials and Methods) which remains after separation of all precipitable anti-ovalbumin. This was shown by electrophoresis not to be homogeneous and to contain the slowest moving components of guinea-pig serum.

Antibody Production in Vitro *by the Local Granuloma, the Homolateral and the Contralateral Flank Lymph Nodes*

As seen from Table II the lymph nodes of the flank on both sides actively incorporated ^{14}C-glycine into anti-ovalbumin during the 4-hr. period of incubation *in vitro*. On the other hand, the granuloma of the injected foot had only 3–10 per cent of the activity shown by the homolateral flank node. Also, it is apparent that the radioactivity of the anti-ovalbumin from the contralateral was 2–3·5 times that of the homolateral flank lymph nodes.

Reference to the histological data (see also Table I) shows that whereas the granuloma of the foot consisted mostly of a proliferation of macrophage elements with only scanty plasma cells, the nodes of both flank regions included large numbers of antibody-containing plasma cells (Plates II and III). Moreover the density of such plasma cells was greater in the contralateral than in the homolateral flank nodes. These facts are compatible with the conclusions that the macrophages do not produce antibody, and that the demonstrated *in vitro* antibody production is in direct relation to the density of antibody-containing plasma cells in these tissues.

Incorporation of ^{14}C-glycine into the γ-globulin fraction followed a similar pattern (Table II). Thus the γ-globulin fraction isolated after 4-hr. incubation of the contralateral gland was 2–3 times more radioactive than the γ-globulin of the homolateral flank glands. However, the local granuloma would appear to form the γ-globulin fraction more actively than the anti-ovalbumin, since it was found to have up to 30 per cent of the activity of the same mass of homolateral flank gland.

TABLE II.—*Relative Ability of Homolateral and Contralateral Flank Lymph Nodes and Local Granuloma to form Anti-ovalbumin and γ-Globulin* 'in Vitro'

Radioactivity : (counts/min./sq. cm.)/0·1 g. tissue incubated

Expt.	Homolateral lymph node in flank.		Contralateral lymph node in flank.		Foot-pad		Contralateral node / Homolateral node	
	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.	Anti-ovalbumin.	γ-globulin.
1*	384	157	907	450	7·8	33	2·36	2·87
2	170	81	590	230	7·9	31	3·45	2·85
3*	125	90	265	185	—	—	2·12	2·05
4	165	110	350	350	17	33	2·12	3·18

* 2 animals pooled.

Sliced tissues from immunised guinea-pigs were incubated for 4 hr. at 37° in the isotonic salt medium containing 2·8 μc ^{14}C-glycine, glucose and a complete amino acid mixture in a total volume of 4 ml. 5 per cent CO_2/95 per cent O_2 was circulated through the system.

Antibody and γ-Globulin Production by a Variety of Tissues of Immunised Guinea-pigs during a Period of Incubation ' in Vitro '

Table III gives data concerning the relative capacity of unit mass of a variety of guinea-pig tissues to incorporate ^{14}C-glycine into anti-ovalbumin and γ-globulin during 3 hours' incubation *in vitro*.

TABLE III.—*Radioactivity of Antibody and γ-Globulin Fractions after Incubation of Tissue Slices with ^{14}C-Glycine*

Tissue.	Weight of organ (g.).	Weight of tissue incubated (g.).	Radioactivity: (counts/min./sq. cm.) /0·1 g. tissue incubated		Density of antibody-containing cells*
			Anti-ovalbumin.	γ Globulin fraction.	
Homolateral flank lymph node	0·6	0·38	967	203	45·1
Contralateral flank lymph node	0·05	0·03	815	300	31·7
Cervical lymph node	0·1	0·08	880	632	15·2
Lumbar lymph node	0·3	0·2	39	23	6·4
Spleen	0·7	0·6	53	133	2·4
Lung	2·6	0·99	2·3	22	0·8
Red bone-marrow	3[4]	0·17	370	425	1·8
Kidney	3·8	1·0	2·5	26	0·1
Liver	16·4	1·0	2	<2	0·1
Thymus	0·25	0·21	—	9	0·1
Placenta	2·0	0·99	—	<2	0
Local granuloma in foot pad	>0·5	0·45	55	73	1·4

* Expressed as numbers per unit microscope field (0·0324 sq. mm.) of 4 μ frozen section.
[4] Approximate figure—taken as about 1 per cent of body weight.

These results are the mean of two experiments with guinea-pigs 79 and 80, immunised in foot pad. The minced organs were incubated *in vitro* for 3 hr. at 37° in an isotonic salt medium containing 0·3 per cent glucose, 1·7 mg. complete amino acid mixture and 2·5 μc ^{14}C-glycine.

Different weights of tissue were incubated, depending on their availability and activity. Thus more of the inactive tissues was incubated in order to increase radioactivity to more easily recordable levels. The results of the radioactivity determinations using different tissues are made comparable by expressing radioactivity of the protein in counts/minute/sq. cm. for 0·1 g. of tissue incubated. It had previously been established that the radioactivity of the protein formed was proportional to the weight of tissue incubated. Activity is also recorded as a percentage of that shown by the homolateral flank lymph nodes (Fig. 3).

The different tissues showed a wide range of activities. The highest radioactivity measurements per unit weight of tissue were attained by a range of lymph nodes, all of which are remote from the site of injection. The low activity of the lumbar nodes correlated with their relatively low content of antibody-containing plasma cells. These nodes are enlarged, but this is due to proliferation of macrophage elements. As described in the histological section, the other remote lymph nodes all contain dense proliferations of antibody-containing plasma cells. The bone marrow incorporated ^{14}C-glycine into anti-ovalbumin at one-third of the rate of the same mass of homolateral flank lymph node. Some activity was manifested by the spleen, but all other organs tested (lungs, liver, kidney, placenta and thymus) showed a negligible amount of incorporation of ^{14}C-glycine into the anti-ovalbumin fraction.

The results of counting antibody cells within appropriately stained sections of samples of the above tissues are shown in the last column of Table III. A fairly good correlation was shown in most instances between the *in vitro* activity of the tissue in the incorporation of radioactive amino acids into the anti-ovalbumin and the density of antibody-containing cells.

The general pattern of relative activities of the various tissues in incorporating ^{14}C-glycine into the γ-globulin fraction is similar to the above (Fig. 3) . On the whole tissues which can form specific antibody also appear to form γ-globulin. The interpretation is somewhat limited by the lack of homogeneity in this fraction, and only broad conclusions are justified. It would appear that certain tissues form γ-globulin more actively than anti-ovalbumin (Fig. 3). This is strikingly apparent in the cervical lymph nodes, the local granuloma, the spleen and bone marrow.

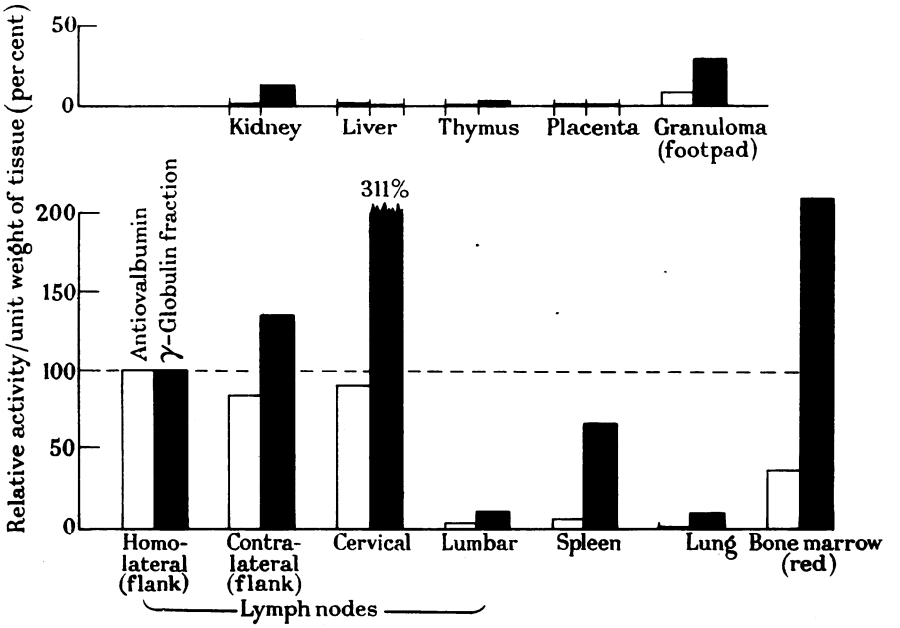


FIG. 3.—Data from guinea-pigs No. 79 and 80 (Table III) showing the relative ability of various tissues to incorporate ^{14}C-glycine into anti-ovalbumin and γ-globulin fractions *in vitro*. White columns : anti-ovalbumin ; black columns : γ-globulin fraction. Tissues incubated for 3 hours at 37° in isotonic medium containing glucose, amino acid mixture and 2·5 μc of ^{14}C-glycine. Activity is represented by radioactivity of the protein fractions (counts/min./sq. cm. at infinite thickness) per 0·1 g. of tissue incubated. The activity of the homolateral flank lymph node was taken arbitrarily to represent 100 per cent activity.

Fig. 4 gives the result of another experiment with a greater number of lymph nodes. Some variation occurs from animal to animal in the relative activities of certain nodes. Thus if the various activity figures are referred to the arbitrarily chosen standard of the homolateral flank nodes some variation is apparent in the relative activities of the contralateral flank and cervical lymph nodes. The activity per 0·1 g. incubated contralateral flank node was strikingly high in the experiment shown in Fig. 4 : the activity of the homolateral popliteal node was very low. The latter is the regional lymph node which immediately drains the injected foot with its depot of antigen. The appearance of tissue sections was one of an intense proliferation of macrophages which almost completely excluded the normal

lymphoid elements, and antibody-containing plasma cells were extremely few.

The results of counting antibody-containing cells within appropriately treated sections of samples of the above tissues are shown in Table IV. Having regard to the inaccuracies of the method, which were particularly increased in the very small lymph nodes weighing less than 0·2 g., and of which only a small part could be spared for a histological investigation, the figures for the counts of antibody-containing cells reproduce broadly the pattern of the *in vitro* activities. However,

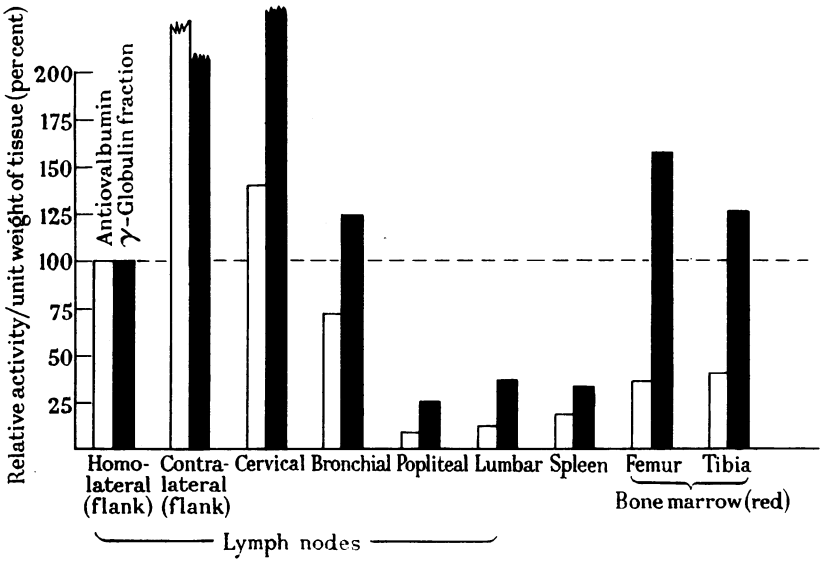


FIG. 4.—Data similar to those in Fig. 3 from guinea-pig No. 144.

the bone marrow appears more active *in vitro* than would be expected from the density of antibody-containing plasma cells. Thus in the experiments of Fig. 3 and 4 the bone marrow had more than one-third the activity of the homolateral flank lymph node and only 5–20 per cent of the number of antibody-containing plasma cells. Compared with the spleen the bone marrow contained fewer plasma cells and yet was 7 times as active *in vitro*.

TABLE IV.—*Density of Antibody-containing Plasma Cells in Various Tissues*

Number of cells per microscope field (area 0·0324 sq. mm.) Guinea-pig No. 144.

Homolateral flank lymph node	28·0
Contralateral flank lymph node (2)	42·3, 59·2
Cervical lymph node	29·2
Popliteal lymph node	0·9
Lumbar lymph node	1·4
Spleen	10·8
Bone-marrow (femur)	5·4

Estimation of the Relative Contributions made to the Production of Anti-ovalbumin and γ-Globulin by a Variety of Guinea-pig Tissues

Fig. 5 is an attempt to express the total activity of different tissues within an immunised guinea-pig, by taking into consideration the *in vitro* activity per unit weight of tissue as given in Table III and the total weight of tissue in the animal. The accuracy of this is limited by the fact that not all organs were studied. Also

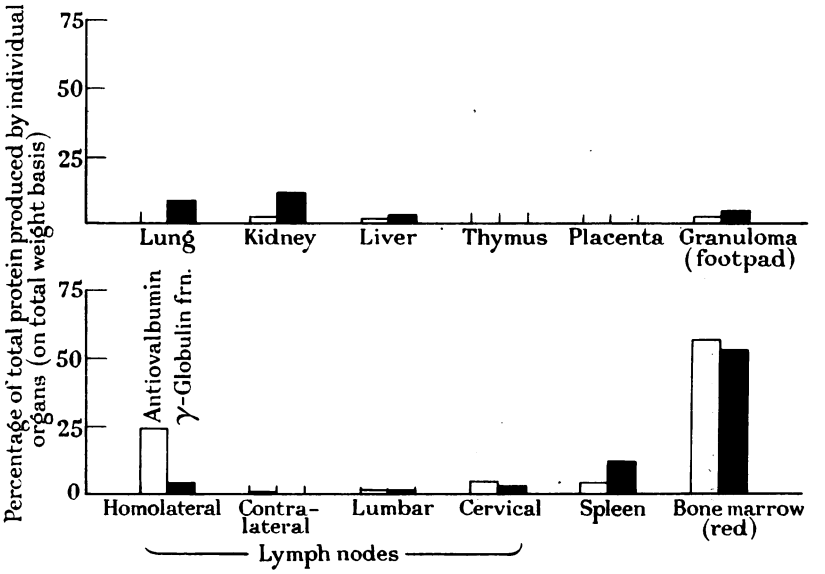


FIG. 5.—The relative proportion of anti-ovalbumin and γ-globulin fraction produced by individual organs. Black columns : γ-globulin fraction ; white columns : anti-ovalbumin. Calculations are based on experimental findings described in Table III. Results are expressed as percentage of total anti-ovalbumin and γ-globulin produced by each of all the organs tested, on basis of its total weight and relative *in vitro* activity per 0·1 g. of tissue incubated.

bone marrow contains differing proportions of red and yellow marrow in various physiological conditions. The figure of 1 per cent of body weight taken here is based on the work of Nye (1931) who estimated that the red bone marrow formed 1 and 1·7 per cent of the total body weight of human beings and rabbits respectively.

As shown by Fig. 5, the main contribution to the total anti-ovalbumin production of the guinea-pig is made by the lymph nodes of the flank and by the bone marrow. The cervical lymph nodes and the spleen add a small amount (less than 10 per cent). The remaining tissues studied make negligible contributions or none at all.

DISCUSSION

The capacity of a variety of different lymph nodes, on a unit weight basis, to synthesise anti-ovalbumin *in vitro*, varied over a wide range of values and this could be correlated with the numbers of antibody-containing cells within these. Since such cells are identifiable as members of the plasma cell family (White *et al.*, 1955) further evidence is provided for the rôle of these cells as producers of specific antibody globulin.

There has been much discussion whether incorporation of a ^{14}C-amino-acid into a protein *in vitro* represents protein synthesis or whether the protein becomes radioactive by adsorption on existing protein or by a non-specific exchange reaction. Granuloma tissue from rabbits immunised with ovalbumin was found (Askonas and Humphrey, 1955) to incorporate ^{14}C-glycine into the anti-ovalbumin during incubation *in vitro*, but not into rabbit pneumococcal antibody added at the start of incubation. Thus it may be taken that incorporation of ^{14}C-amino-acids into the soluble antibody and γ-globulin fractions occurs only when the incubated tissue is producing these proteins.

The numerous studies based on extraction of antibody from tissues after immunisation procedures all possess the disadvantage of possible confusion between local production of antibody and local concentration of antibody formed elsewhere. One is left wondering whether the studies of Westwater (1940*a*,*b*) and Freund, Schryver, McGuiness, and Geitner (1952), showing high concentrations of antibody at the local site of injection of antigen in Freund-type adjuvant mixtures, really denote local production of antibody. There is a suggestion from the work of Westwater (1940*a*) that local concentration was actually occurring, for in some animals from which the local lesions had been removed antibody appeared sooner in the circulation than in controls in which the lesion was left intact, *i.e.*, in the latter the local lesion appeared to function by extracting antibody from the circulation until it was saturated. In the present animals the 3-week-old lesions of the foot-pad showed much extracellular antibody associated with adventitia of blood vessels and collagen but very few antibody-containing cells (or plasma cells).

The present findings are that the local granuloma in the guinea-pig under the condition of immunisation had a low relative *in vitro* activity and would be expected to contribute little to the animal's production of antibody. In the rabbit, the local granuloma produced by Freund's adjuvant possesses a low activity per unit weight of tissue when compared with the antibody-synthesising activity of lymph nodes ; nevertheless, on the basis of its total weight the granuloma appears to contribute an appreciable proportion of the antibody produced by the whole animal (Askonas and Humphrey, 1955). It is possible that conditions such as dosage of antigen, species of experimental animal and site of injection may greatly influence the rate of production of antibody at the local site of injection of antigen, and the proportion which this contributes to the antibody produced in the whole animal.

The low numbers of antibody-containing cells found in the other tissues of the body (liver, lungs, kidney, spleen, thymus) correlated well with the low or negligible *in vitro* activity for anti-ovalbumin synthesis, with the exception of the bone-marrow. The few experiments which have included the study of this tissue have failed to show as many plasma cells or antibody-containing cells as would be expected from the relatively high *in vitro* activity. This discrepancy is being investigated. The technical difficulties of the morphological studies are greater with the bone marrow than with other tissues. The matter is of some importance, since by assuming the weight of bone marrow to equal about 1 per cent of the total body weight, this tissue could be regarded as an important site of the antibody formation of the whole animal.

While the present investigation provides evidence that specific antibody is formed by members of the family of plasma cells, the cellular site of production of γ-globulin is still far from clear. According to the Mudd-Haurowitz hypothesis the

presence of antigen diverts the activities of the cell normally producing γ-globulin to form specific antibody. Since the numerous plasma cells found in immunised animals make their appearance in response to the specific stimulus of antigen the production of " normal " γ-globulin as envisaged by the Mudd-Haurowitz theory cannot apply to these newly differentiated cells. The reconciliation of this theory with morphological facts is only possible if a precursor of plasma cells is responsible for synthesis of " normal " γ-globulin and if the morphological change implicit in the development of the family of plasma cells is a manifestation of increased rate of protein synthesis. Unfortunately the nature of the precursor of the plasma cell family is obscure.

Alternatively, the plasma components classed as " normal " γ-globulin may consist of a collection of small amounts of specific antibody (Hughes, 1954). The γ-globulin fraction is known to be heterogeneous (Porter, 1955). The morphological implication of this hypothesis would be that all γ-globulin production would be associated with plasma cells. This view receives support from the findings that patients suffering from agammaglobulinaemia do not form plasma cells when stimulated with antigens (Good, 1955 ; Gitlin, 1955) which suggests that the essential defect of agammaglobulinaemia is located in the same system of cells.

Conclusions about γ-globulin formation based on the present results are limited by the nature of antigen administration and by the impurity of the γ-globulin fraction. They do show, however, that no γ-globulin is synthesised by the liver. This supports the evidence of Miller and Bale (1954) obtained by the use of perfused liver preparations. Similarly, thymus and placenta show no activity. However, many other tissues appear to contribute to the production of γ-globulin to varying extents. On the whole, formation of the γ-globulin fraction in various tissues follows a pattern similar to that found for the synthesis of anti-ovalbumin. Tissues such as the homolateral and contralateral lymph nodes of the flank, the cervical lymph nodes and the bone marow, which actively incorporate ^{14}C-glycine into anti-ovalbumin, are also active in forming the γ-globulin fraction (which contains the γ-globulin components not precipitable with ovalbumin.)

The low *in vitro* activity of the local granuloma, and of the homolateral popliteal and lumbar lymph nodes, which in these animals are largely composed of macrophages, would certainly indicate that the latter are not responsible for production of γ-globulin. Furthermore it is striking that certain organs can incorporate the radioactive amino acid more rapidly into the γ-globulin fraction than into the anti-ovalbumin (*i.e.*, cervical lymph nodes, bone marrow and spleen). These tissues must be important contributors to the production of γ-globulin found normally in the plasma.

SUMMARY.

Guinea-pigs were injected in the foot-pad with a single dose of ovalbumin in a Freund-type adjuvant mixture and three weeks afterwards various tissues were examined for their content of antibody-containing plasma cells and their ability to incorporate ^{14}C-glycine into the anti-ovalbumin and the remainder of the γ-globulin during a period of incubation *in vitro*.

The granuloma at the site of injection of the antigen mixture, and the immediate draining lymph node (popliteal) which were composed mostly of macrophages and had few antibody-containing plasma cells were relatively inactive in the synthesis of anti-ovalbumin *in vitro*.

The lymph nodes more remote from the site of injection were extremely active in incorporating ^{14}C-glycine into the anti-ovalbumin. In most cases, the highest activity was found in the lymph nodes of the contralateral flank.

Organs other than lymph nodes had low *in vitro* activity with the exception of the spleen and bone marrow. If this activity of the bone marrow were uniform throughout the red marrow of the whole animal this would be a major contributor to the total antibody.

A good correlation existed throughout the lymph nodes and the other organs tested (with the exception of the bone marrow) between the number of antibody-containing plasma cells seen in tissue sections and the ability of unit weight of the different tissues to form anti-ovalbumin *in vitro*.

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