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The Role of Mycobacteria and Silica in the Immunological Response of the Guinea-Pig

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Summary. The relationships between the development of a local epithelioid or macrophage granulomatous tissue reaction, of delayed-type hypersensitivity and of biosynthesis of γ_2-immunoglobulin have been explored in guinea-pigs injected with antigen mixtures containing aluminium silicate (bentonite) particles in saline suspension or containing whole mycobacterial cells or their extracted peptidoglycolipids in water-in-oil emulsions.

Bentonite was found to be effective in inducing local granulomatous tissue proliferation and the formation of γ_2-immunoglobulin. In the same animals, in a minority of circumstances, bentonite was capable of inducing delayed-type hypersensitivity to the accompanying protein antigen. Thus bentonite could reproduce, in these three respects, the specific immunological effects produced by mycobacteria or their extracted peptidoglycolipids. By the use of prior injection of the homologous antigen, partial blockage of the production of delayed-type hypersensitivity following subsequent injection of the antigen in Freund's complete adjuvant could be effected. At the same time changes occurred in granuloma formation and biosynthesis of γ_2-immunoglobulin. The reasons for the apparent linkage of these events are discussed.

INTRODUCTION

Guinea-pigs injected into the footpad with a single dose of ovalbumin or other protein antigens in an adjuvant mixture consisting of a water-in-oil emulsion with added heat-killed *M. tuberculosis* produce antisera 3 weeks later which contain precipitating antibody of fast (γ_1) and slow (γ_2) mobility on immunoelectrophoresis (White, Jenkins and Wilkinson, 1963; Benacerraf, Ovary, Block and Franklin, 1963). If the mycobacteria are omitted from the adjuvant mixture, the slow (γ_2) anti-ovalbumin component is not produced and newly synthesized antibody appears only in the γ_1 position. Neither is γ_2-immunoglobulin antibody produced after a single footpad injection of a protein antigen mixed with other non-mycobacterial adjuvants such as aluminium phosphate. Similarly, the inclusion of mycobacteria in Freund's adjuvant mixtures is necessary for the development of delayed-type hypersensitivity to the injected antigen as judged by the corneal response to a test dose of antigen 21 days later (White, Coons and Connolly, 1955).

In an attempt to explore more fully the circumstances under which guinea-pig γ_1- and γ_2-anti-ovalbumin are synthesized, the antigen (ovalbumin) was injected by different routes into the guinea-pig together with a number of non-mycobacterial adjuvants, the

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chief of which was bentonite, the native colloidal form of aluminium silicate. Evidence for its activity as an adjuvant in the guinea-pig is presented and its effect on the synthesis of γ_1- and γ_2-antibodies is described. In further experiments, the immunological response to ovalbumin in complete Freund-type adjuvant was blocked by a previous injection of ovalbumin in mineral-oil. This latter manoeuvre was an attempt to block the development of delayed-type hypersensitivity using a method suggested by Boyden (1957). This author showed that injection of tuberculoprotein 2 weeks before the injection of BCG live vaccine prevented the normal development of delayed-type hypersensitivity as tested by an intradermal injection of tuberculoprotein (Mantoux test).

The guinea-pig γ_2- and γ_1-immunoglobulins under discussion have non-identical heavy chains and are antigenically distinct from one another (Askonas, White and Wilkinson, 1965). Although they show resemblances to the human immunoglobulins γG and γA, guinea-pig immunoglobulins differ in certain biochemical properties and in biological activity from their human analogues and the designation γ_2 and γ_1 for the slow and fast 7S immunoglobulins in the guinea-pig has therefore been retained in this communication.

MATERIALS AND METHODS

Ovalbumin

Ovalbumin was prepared from pooled egg-white by the method of Rhodes, Azari and Feeney (1958) using elution from a carboxymethylcellulose column (Whatman, CM.70) in acetic acid–ammonium acetate buffer. The fractions collected by elution with 0·1 M acetate buffer, pH 4·6, were pooled, dialysed against running tap water and lyophilized. Cellulose acetate electrophoresis of this preparation showed a strong bifid band in the ovalbumin position and a trace of slower-moving protein present as an impurity. As immunization of guinea-pigs with this preparation yielded antisera containing antibodies against ovalbumin alone and not against the impurity, the preparation was accepted as satisfactory for use as an antigen in immunization experiments.

Human serum albumin (HSA)

Human serum albumin was obtained from Behringwerke A.G., Marburg, Lahn. Freeze-dried 'Human-albumin, trocken, reinst' was used as an antigen for guinea-pig injection.

Rabbit anti-guinea-pig globulin

Antisera against guinea-pig globulin were prepared in rabbits as described by Stewart-Tull, Wilkinson and White (1965).

Bentonite

Bentonite is a native, colloidal, hydrated aluminium silicate, the principal constituent being montmorrilonate, $Al_2O_3.4SiO_2.H_2O$ (Martindale, 1952). It is an insoluble buff powder whose properties as an adsorptive agent for soluble proteins are well known and have been used for the *in vitro* detection of antigens by the bentonite fixation text (Bozicevich, Bunim, Freund and Ward, 1958). It was obtained as a powder from British Drug Houses Ltd and contained aluminium silicate with 2 per cent of sodium and 2 per cent of calcium present as impurities. For use as an adjuvant, 0·25 g of bentonite powder was shaken up in 5 ml saline. The suspension was allowed to stand for 10 minutes during which time the heavier particles settled to the bottom of the tube. The supernatant fluid containing the lighter bentonite particles in suspension was then decanted to another tube.

Appropriate weighed amounts of lyophilized ovalbumin were added to the suspension for use. The bentonite-adsorbed ovalbumin was injected not less than 30 minutes after mixing.

Different batches of guinea-pigs were injected with bentonite-adsorbed ovalbumin intraperitoneally and into the footpad. For intraperitoneal injection, 1·5–2 mg ovalbumin were made up in 0·25 or 0·5 ml of the bentonite suspension and injected into each guinea-pig, either at intervals of 4 days or weekly for up to 3 weeks, giving the first injection into the left side of the abdomen, the second into the right and so on. Guinea-pigs were killed and bled at intervals of between 14 days and 8 weeks after the primary injection. Footpad injections were given at the same time intervals, again in alternate hind footpads. Two milligrams of ovalbumin was injected in 0·2 ml bentonite suspension, this being the maximum volume of the thick bentonite suspension that it was usually possible to inject into the guinea-pig footpad.

Mycobacteria

Mycobacteria which were used to prepare complete Freund's adjuvant mixtures were *Mycobacterium tuberculosis*, strain C, a heat killed human-type organism supplied by the Ministry of Agriculture and Fisheries, Weybridge, Surrey. In some complete adjuvant mixtures, peptidoglycolipid of human-type *Mycobacterium tuberculosis*, strain Canetti, was used instead of whole cells of *M. tuberculosis* (White, Jolles, Samour and Lederer, 1964).

Immunization procedure for blocking the development of delayed hypersensitivity in response to ovalbumin in complete Freund's adjuvant

Ovalbumin was made up as a solution in physiological saline (50 mg in 1·0 ml) and incorporated in a water-in-oil emulsion without mycobacterial components. The proportions of this mixture by volume were; saline solution of antigen, 1·0: arlacel A, 1·0: mineral oil (Bayol F), 3·0. Guinea-pigs were injected in the left hind footpad with 0·2 ml of the well-emulsified mixture and approximately 15 days later were re-injected into the right hind footpad with 0·2 ml of a water-in-oil emulsion of ovalbumin (2 mg per guinea-pig) in complete Freund's adjuvant mixture containing 1 mg of heat killed *M. tuberculosis* strain C per animal. In some experiments, the mycobacteria were replaced by the same weight of peptidoglycolipid of *M. tuberculosis* from the same strain (White *et al.*, 1964). These animals were tested by intracorneal injection of ovalbumin 19 days later and were killed at 21 days.

The above procedure was modified slightly for blocking of development of delayed hypersensitivity to HSA. Three preliminary injections of 2 mg of HSA were given in physiological saline into alternate footpads at daily intervals. After an interval of 15 days, 2 mg of HSA was given *via* the footpad in a water-in-oil emulsion containing 500 μg of heat-killed *M. tuberculosis* strain C.

Measurement of local granulomatous reactions in the injected guinea-pig foot

The site of injection of complete Freund-type adjuvant mixture in the footpad is characterized by the development of a firm swelling 7–14 days later which subsequently ulcerates at 15–21 days. This granulomatous swelling was measured at the point of maximum plantar-dorsal thickness of the injected foot with a Schnelltaster callipers (Carbronze Ltd, Belmont Road, London, W.4).

Immunoelectrophoretic analysis of guinea-pig sera

All guinea-pig sera were analysed by immunoelectrophoresis in 0·8 per cent Ionagar, No. 2 (Oxoid) in barbitone buffer, 0·05 M, pH 8·4. After electrophoresis, the trough

on one side of the separated serum protein bands was filled with ovalbumin at a strength of 100 μg/ml in phosphate-buffered saline, pH 7·2. The trough on the other side was filled with rabbit anti-guinea-pig globulin serum. Precipitin arcs were allowed to develop for 36 hours and the plates were then washed and stained.

Quantitative estimation of antibody

The total antibody nitrogen content of guinea-pig sera was estimated by measurement of the extinction at 280 mμ of the washed precipitate obtained at optimal proportions with antigen. The precipitate suspended in 1 ml of saline was re-dissolved by addition of 0·1 ml of 0·02 M KOH. Antibody nitrogen was then estimated by comparison with a standard curve for extinction for known weighed amounts of guinea-pig γ-globulin.

Separation of guinea-pig γ-$_1$ and γ_2-antibodies

The fast and slow guinea-pig γ-globulin fractions were separated on DEAE-cellulose columns (Whatman, DE 50) using a cone and sphere gradient from 0·01 to 0·3 M phosphate. The technique used was described by Stewart-Tull *et al.*, (1965). After concentration of the protein fractions against polyethylene glycol (Carbowax 20 M, G. T. Gurr), their anti-ovalbumin content was checked by immunoelectrophoresis against ovalbumin as described.

Corneal reactions

The corneal reaction of guinea-pigs immunized with ovalbumin was tested by injecting a blob of a solution of ovalbumin at a strength of 20 mg/ml under the surface of the edge of the cornea. The degree of opacity was observed 24 hours after injection. The corneal reactions were tested 17–21 days after primary immunization in all cases. Corneal reactions were scored as follows: 0, no change; 1, slightly increased opacity in the area around the needle injection; 2, greater opacity covering most of the cornea; and 3, great thickening and grey opacity of the whole extent of the cornea accompanied by chemosis of the conjunctiva.

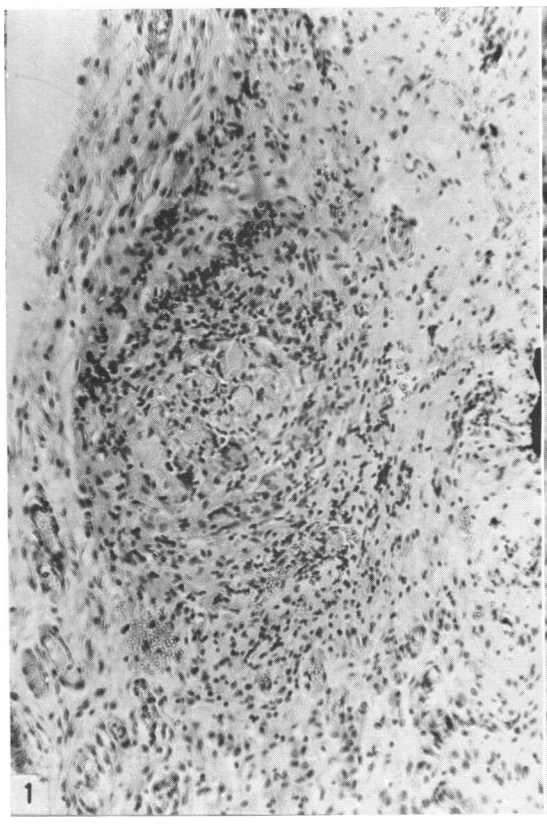
Fluorescent staining of guinea-pig tissues

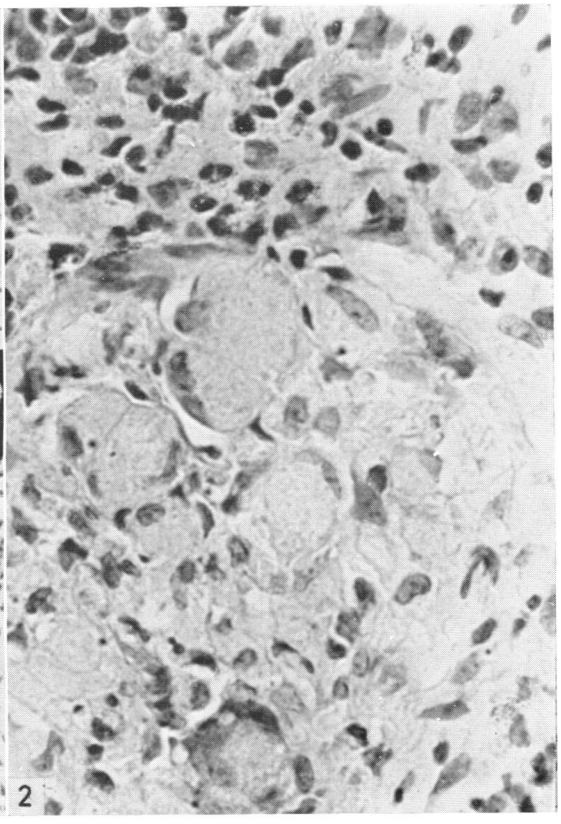
Specimens of the spleen, mesenteric lymph nodes and local granulomata from guinea-pigs injected intraperitoneally with bentonite-adsorbed ovalbumin were taken into acetone–dry ice mixture. These tissues were stored at $-20°$ and sections were made from them in a cryostat. The sections were fixed for 15 minutes in absolute methanol. After re-hydration, they were treated with a solution of ovalbumin at a strength of 1 mg/ml for 30 minutes, washed in buffered saline, pH 7·2 and stained for 30 minutes with fluorescein-conjugated anti-ovalbumin. After washing, they were mounted in buffered glycerol and examined immediately using a Leitz microscope with HBO 200 mercury vapour lamp as light source and BG 12 barrier and OG 9 eye-piece filters. A dark ground condenser was used.

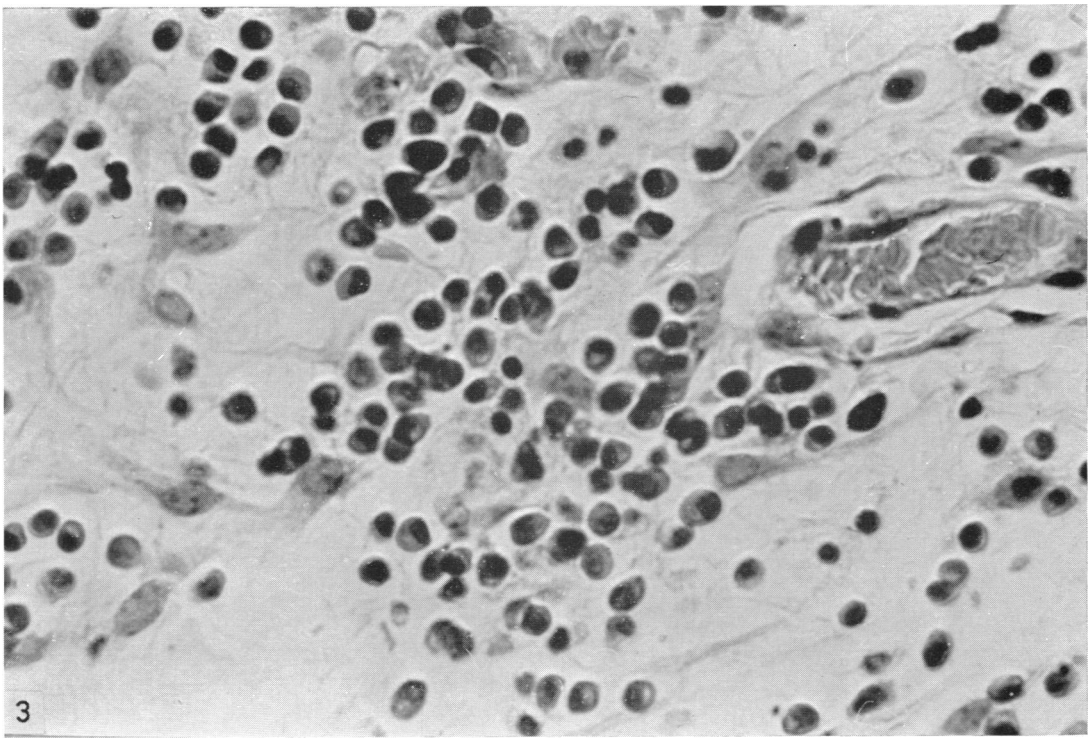
FIG. 1. Peritoneal granuloma in a guinea-pig injected with bentonite-adsorbed ovalbumin. A central giant-cell granuloma is surrounded by a ring of macrophages and lymphocytes. H & E. × 60.

FIG. 2. High-power view of part of the granuloma shown in Fig. 1, again showing that the granuloma is made up of a central area of giant cells and macrophages with lymphocytes, macrophages and eosinophils in the surrounding ring. × 270.

FIG. 3. A group of plasma cells present in newly formed fibroblastic tissue in a peritoneal adhesion from a guinea-pig injected with bentonite-adsorbed ovalbumin. H & E. × 320.







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RESULTS

HISTOLOGICAL FINDINGS AFTER INTRAPERITONEAL IMMUNIZATION OF GUINEA-PIGS WITH BENTONITE-ADSORBED OVALBUMIN

Guinea-pigs were given weekly intraperitoneal injections of bentonite-adsorbed ovalbumin and were killed at intervals between 2 and 8 weeks after commencement of the course of injections. Necropsies were performed on all guinea-pigs. Examination of the peritoneal cavities revealed the following lesions: in the site of the most recent injections made 7–14 days before necropsy, there was congestion around the site of injection with scattered soft yellow nodules in the immediate vicinity. These nodules contained a soft necrotic material sterile on bacteriological examination. Microscopically, the early lesions showed a central amorphous, necrotic area surrounded by a densely cellular ring of macrophages and polymorphs with some lymphocytes. Many degenerate-looking cells were seen. In older lesions at the site of injections carried out 14–28 days previously, fibrous tissue reactions were prominent. Dense adhesions were seen between the parietal surface of the peritoneum near the injection site and the contiguous visceral peritoneum and the omentum. Scattered firm grey plaques were also seen on the peritoneal surface. These lesions showed the histological picture of a macrophage granuloma. Scattered localized granulomata were seen in which a central area of pale eosinophil giant cells with peripheral nuclei was often present. Surrounding these were macrophages and lymphocytes (Figs. 1 and 2). There was an intense fibroblastic reaction at the site of the adhesions. Contained in the new fibrous tissue and often in the vicinity of newly formed capillaries were numerous small foci of plasma cells (Fig. 3). Plasma cells were more commonly seen in this site than in the macrophage granuloma itself where their presence was exceptional.

The granulomatous and fibroblastic lesions in the injection area as well as the draining mesenteric lymph nodes, which were usually enlarged, and the spleen were examined by the fluorescent antibody technique for the presence of antibody using a double-layer method with ovalbumin as the first layer and fluorescein labelled anti-ovalbumin as the second layer, and for antigen using a single layer of fluorescein labelled anti-ovalbumin. Large amounts of antigen were detected free and within macrophages at the injection site. Macrophages in the draining lymph nodes also contained antigen. Anti-ovalbumin containing cells could be seen in the mesenteric lymph nodes and in the peritoneum at the site of injection but in moderate quantities only. Considerable variation was found between individual animals studied. Anti-ovalbumin containing cells were also found in small numbers in the spleen.

The presence and the extent of the granuloma in bentonite-injected guinea-pigs was very variable. Good reactions of the type described above occurred in slightly over half of the animals injected. In other animals, granuloma and adhesion formation was slight or, in some animals, absent. In the latter group, plasma cells could not be detected at all in the vicinity of the lesions; neither could antibody forming cells be detected in the local lymph glands. The variability in the extent of granuloma formation was quite unpredictable. Different animals from the same batch sometimes showed marked granuloma formation, sometimes none at all.

ANTIBODY PRODUCTION IN BENTONITE-INJECTED GUINEA-PIGS

Immunoelectrophoretic patterns

The sera from guinea-pigs killed 14 days after the commencement of immunization with bentonite-adsorbed ovalbumin contained no detectable antibody. However, immuno-

TABLE 1

GRANULOMA FORMATION, IMMUNOGLOBULIN PRODUCTION AND CORNEAL REACTIONS IN GUINEA-PIGS INJECTED WITH BENTONITE-ADSORBED OVALBUMIN

Guinea-pig No.	Granuloma	Adhesions	Serum immunoglobulin		Corneal response
			γ_1 present	γ_2 present	
(1) Intraperitoneally injected group killed at 28 days					
5217	++	Present	+	+	1½
5231	+++	Present	+	+	1
5396	+	Present	+	+	1
5398	++	Present	+	+	3
5399	+++	Present	+	+	2
5392	++	Present	+	+	N/T
5395	++	Present	+	−	0
5397	++	Present	+	−	1½
5528	+	Absent	+	−	1
5218	−	Absent	−	−	0
5232	−	Absent	−	−	0
5394	−	Slight	−	0	−
5532	+++	Present	N/T		0
5533	++	Absent			0
5523	+	Absent			2
(2) Footpad injected group killed at 21 days					
5227	++		+	−	0
5228	++		+	−	N/T
5229	++		+	−	N/T
5230	++		+	−	N/T
5393	++		+	−	0

N/T, Not tested.

electrophoretic analysis of the sera from animals killed at 21 or 28 days after primary injection revealed a double anti-ovalbumin arc of the type described by White *et al.* (1963) using mycobacterial adjuvants (Table 1). In six out of twelve 28-day sera, the γ_1- and γ_2-anti-ovalbumin components were both clearly visible (Fig. 4). In three animals a fast (γ_1) arc only was present. Three sera failed to show anti-ovalbumin arcs at all; two of these sera were taken from guinea-pigs that had failed to show the formation of peritoneal adhesions or granulomata at the injection site and in the third animal, such reactions had been minimal. On the other hand, the animals whose sera contained both anti-ovalbumin arcs all also showed prominent local tissue reactions to the injected material with granuloma formation and the presence of plasma cells in recently formed fibroblastic tissue. Similar results were found in guinea-pigs killed 21 days after commencement of immunization, but batches of guinea-pigs killed at 6 and 8 weeks—the latest boost having been given at 21 days—showed no antibody formation although peritoneal granulomata were present.

Quantitative precipitin titres

Table 2 shows the results of quantitative precipitin tests on sera from guinea-pigs injected intraperitoneally with bentonite-adsorbed ovalbumin. In terms of precipitin levels, bentonite was about as effective as an adjuvant as water-in-oil emulsions lacking in mycobacteria. Precipitin levels were certainly lower than those found using mycobacterial adjuvants. On the other hand, bentonite was more effective than incomplete Freund's adjuvant in stimulating production of γ_2-globulin anti-ovalbumin. The scatter of precipitin

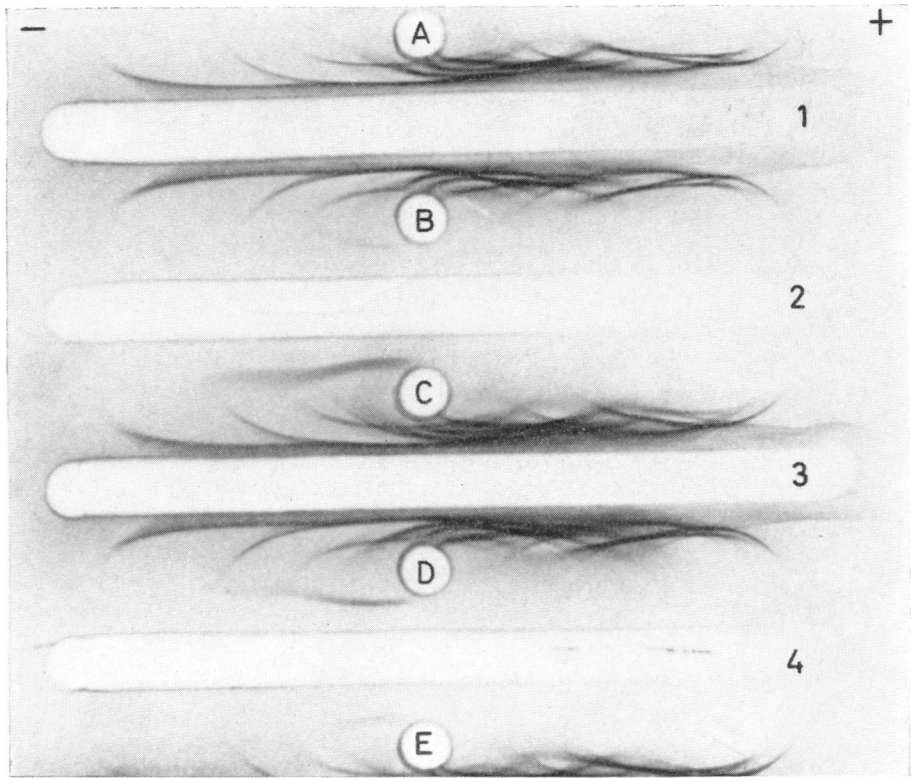

FIG. 4. Agar gel immunoelectrophoresis of sera from guinea-pigs injected with bentonite-adsorbed ovalbumin. The wells contained: A and B, sera from guinea-pigs 5230 and 5229 injected via the foot-pad; C, D and E, sera from guinea-pigs 5217, 5231 and 5397 injected intraperitoneally.

Troughs 1 and 3 contained rabbit anti-guinea-pig globulin and troughs 2 and 4 contained ovalbumin (100 μg/ml). Note that while sera A and B (footpad injected) show only a fast (γ_1) anti-ovalbumin arc, sera C and D (intraperitoneally injected) show both fast (γ_1) and slow (γ_2) anti-ovalbumin arcs. Serum E shows only a fast (γ_1) arc.

TABLE 2

CORRELATION OF PRECIPITIN TITRES USING: (a) INTRAPERITONEAL INJECTION OF BENTONITE-ADSORBED OVALBUMIN, AND (b) FOOTPAD INJECTION OF COMPLETE AND INCOMPLETE FREUND'S ADJUVANT IN THE GUINEA-PIG

Adjuvant	No. of animals	Preciptitin level in serum (μg N/ml) (mean)	Standard deviation
Bentonite	10	169	±136
Incomplete Freund	21	113	±76
Complete Freund	34	284	±102

levels in the bentonite-injected group was high, some guinea-pigs producing no antibody at all and others showing double anti-ovalbumin arcs and quite high precipitin titres. However, antibody production appeared to be related both quantitatively and qualitatively to the success or failure to stimulate a macrophage granuloma at the injection stite.

Delayed hypersensitivity

Corneal reactions were tested at 21 days after primary intraperitoneal injection. Most guinea-pigs injected with bentonite showed either negative or weakly positive corneal responses (Table 1). Strong reactions were seen in only three out of fifteen animals, two of which showed the presence of γ_2-anti-ovalbumin in the serum and marked granulomatous lesions in the peritoneum. While animals injected with bentonite therefore did show evidence of delayed sensitivity, the relationship between delayed hypersensitivity and the production of γ_2-globulin was not as obvious as when mycobacterial adjuvants were used.

Properties of the fast and slow immunoglobulins separated on DEAE-cellulose

The γ_1- and γ_2-anti-ovalbumin fractions from guinea-pigs injected with bentonite were successfully separated from one another by DEAE-cellulose chromatography. The chromatographic behaviour of these fractions was identical to that described for the γ_1 and γ_2 fractions obtained from the sera of guinea-pigs injected with ovalbumin in mycobacterial adjuvants (White *et al.*, 1963). Immunoelectrophoretic analysis revealed γ_1 and γ_2 fractions in the same chromatographic positions as previously described, with no antibody in later fractions where macroglobulin would be likely to be seen. The sera of guinea-pigs immunized with bentonite-adsorbed ovalbumin contained complement-fixing antibodies against ovalbumin. DEAE-cellulose chromatography and subsequent examination of the fractions obtained revealed that these antibodies were confined to the γ_2 fraction. PCA activity was found in the γ_1 fraction from both bentonite-injected and Freund-injected animals.

Footpad injection of bentonite-adsorbed ovalbumin

Guinea-pigs were injected weekly with bentonite-adsorbed ovalbumin into alternate footpads. These animals were killed and bled 21 days after commencement of the experiment. Considerable swelling of the footpads, usually with the appearance of necrotic abscesses similar to those seen in the peritoneum, was generally observed within a week or two of the commencement of immunization. Histologically, a similar tissue reaction with a predominant macrophage response was seen, as found in animals injected intraperitoneally. Plasma cells were not seen in the footpad lesions. Examination of the sera of this group of animals by immunoelectrophoresis revealed a γ_1 arc alone (Table 1). γ_2-Globulin arcs were not seen after footpad injection of bentonite (Fig. 4) nor was complement fixing antibody found in the serum. The corneal responses were negative in those animals tested.

Other non-mycobacterial adjuvants. Local granulomatous lesions were not seen, neither was circulating antibody detected in groups of guinea-pigs injected intraperitoneally with ovalbumin in aluminium phosphate or in calcium alginate.

INHIBITION (BLOCKING) OF DELAYED-TYPE HYPERSENSITIVITY WITH A PREVIOUS INJECTION OF ANTIGEN IN INCOMPLETE FREUND'S ADJUVANT

Table 3 shows the results of corneal tests, serum immunoglobulin patterns and granuloma dimensions after a footpad injection of ovalbumin in complete Freund's adjuvant (Group A) compared with the same results after attempted inhibition of this response by

TABLE 3

CORNEAL RESPONSES, GRANULOMA AND IMMUNOGLOBULIN PATTERN IN GUINEA-PIGS 'BLOCKED' BY PRIOR INJECTION OF OVALBUMIN AND IN A POSITIVE CONTROL GROUP

Animal	Corneal response	Serum immunoglobulin		Footpad plantar-dorsal dimension (cm)	Ulceration at 3 weeks after injection
		γ_1 present	γ_2 present		
Group B* ('blocked')					
5572	2	+	−	1·8	Absent
5573	2	+	−	1·9	Absent
5574	2	+	+	1·7	Absent
5575	1	+	−	1·6	Absent
5513		Weak	−	1·9	Absent
5517		Weak	−	2·0	Absent
Group A† (Positive controls)					
5618	2½	+	+	2·3	Present
5619	½	+	Weak	2·2	Slight
5620	3	Weak	Weak	2·3	Present
5615	2	+	Weak	2·3	Present
5616	½	−	−	2·0	Present
5617	3	−	−	1·6	Absent
5557		+	+	2·4	Present
5558				2·o	Present
5559		−	−	2·4	Present

* Group B received a preliminary single injection, 15 days before the above procedure, of ovalbumin 2 mg in incomplete adjuvant. At the second injection animals 5572, 5573 received ovalbumin (2 mg) in an adjuvant mixture containing 1 mg heat-killed *M. tuberculosis*. In the remaining animals, whole organisms were replaced by the peptidoglycolipid (1 mg).

† Group A received a single injection of 2 mg ovalbumin in complete Freund's adjuvant mixture containing either 1 mg heat-killed *M. tuberculosis* strain C. (Animals 5618, 5619, 5620) or 1 mg peptidoglycolipid from *M. tuberculosis* str. Canetti (Animals 5615–17, 5557–59).

TABLE 4

CORNEAL RESPONSES TO HUMAN SERUM ALBUMIN IN A GROUP OF 'BLOCKED' GUINEA-PIGS AND IN A POSITIVE CONTROL GROUP

Animal	Corneal response
Group D* (injected previously with antigen)	
5784	1
5785	1
5787	1
5788	1
5789	2
Group C† (positive controls)	
5796	3
5797	3
5798	3
5799	3

* Group D received three preliminary injections of 2 mg of HSA in 0·2 ml physiological saline into alternate footpads at intervals of 1 day followed 2 weeks later by injection of HSA as for Group C animals.

† Group C received a single injection of 2 mg of HSA in complete Freund's adjuvant mixture containing 500 μg of heat-killed *Mycobacterium tuberculosis* strain C Weybridge.

previous injection of the same antigen in incomplete Freund's adjuvant (Group B) (see 'Materials and Methods'). It can be seen that animals of Group B had much less local swelling at the site of antigen injection. The average plantar–dorsal dimension of the granuloma at 3 weeks was 1·8 cm in Group B and 2·2 cm in the control Group A. Animals of Group B also showed less ulceration of the foot granuloma at 3 weeks. Fewer (one out of eight) animals in Group B developed γ_2-immunoglobulin precipitin arcs as contrasted with the control Group A (five out of eight).

Convincing differences were not seen between the two groups in delayed-type reactions measured by the corneal test using ovalbumin as antigen. In later experiments a similar experiment was performed using HSA as antigen. Table 4 shows the results of corneal tests after a footpad injection of HSA in complete Freund's adjuvant (Group C) compared with the same results after attempted inhibition of this response by previous injections, repeated thrice, of 5 mg HSA in physiological saline at 2 weeks previously (Group D). It can be seen that animals of Group D showed much less severe corneal responses to HSA than those of the control Group C.

DISCUSSION

A variety of criteria has been used to assess adjuvant activity. They include the ability to increase serum antibody to levels which are higher than those obtained after injection of the antigen minus the adjuvant, the ability to induce a high level of delayed-type hypersensitivity, the capacity to provoke autoimmune diseases (such as allergic encephalomyelitis) after injection of adjuvant plus tissue antigen, and as now seems clear in the guinea-pig, the ability to alter the immunoglobulin type of the antibody produced. By all these criteria, water-in-oil emulsions containing mycobacteria have a strong adjuvant effect on protein antigens. In the guinea-pig the omission of mycobacteria from such emulsions results in lower specific antibody levels (Fischel, Kabat, Stoerck and Bezer, 1952; White *et al.*, 1955) and the failure to induce γ_2-immunoglobulin production (White *et al.*, 1963) or to stimulate delayed hypersensitivity or to provoke, with homologous brain, allergic disseminated encephalomyelitis (White and Marshall, 1958).

The adjuvant effects of silica were clearly described by Pernis and Paronetto (1962) who showed that immune responses in the rabbit and rat were enhanced by the injection of tridymite, the pseudohexagonal orthorhombic form of crystalline silica. Greater adjuvant effects were obtained if the antigen was injected, not at the same time as the silica but at an interval of 60–90 days after it. Quantitative antibody levels were increased and numerous antibody-containing cells were present in the lymph nodes and spleen. In the present experiments, ovalbumin was injected adsorbed on particles of bentonite (aluminium silicate) and an adjuvant effect was clearly established in respect of both quantitative and qualitative changes in the resulting serum antibody. Considerable variation in this effect was found from animal to animal but, under optimal conditions, bentonite was capable of provoking the formation of γ_2-immunoglobulin anti-ovalbumin and of producing serum antibody levels comparable with those attained using incomplete Freund's adjuvant but not as high as those attained using mycobacterial adjuvants. In addition, certain animals showed evidence of delayed hypersensitivity (positive corneal response). A constant association was noted between these three adjuvant effects and the presence and extent of the macrophage granuloma produced at the site of immunization. A similar correlation has been noted previously (Suter and White, 1954; White *et al.*,

1955) between the extent of development of the macrophage–epithelioid cell granuloma at the site of antigen injection in Freund-type adjuvant and the adjuvant effect in terms of elevated levels of serum antibody and delayed-type hypersensitivity. Injection of mycobacterial extracts which failed to facilitate the development of delayed-type hypersensitivity or to show an adjuvant effect on the serum antibody levels failed to cause a local macrophage–epithelioid cell granuloma. When the mycobacterial component of the injection mixture was decreased in amount, both local granuloma formation and adjuvant effects in delayed hypersensitivity and serum levels of antibody decreased in a parallel manner.

The close association found between the extent of the macrophage granuloma and adjuvant activity suggests possible mechanisms for the action of bentonite in enhancing immune responses. Antigens adsorbed onto inert particles such as silicates are likely to remain at the site of injection longer than similar antigens in solution. They are likely to be ingested by phagocytic cells and to provoke a foreign body type macrophage response. Slow release of antigen from the local granuloma might therefore allow increased stimulation of immunologically competent cells in the local lymph nodes. Allison, Harington, Birbeck and Nash (1965) have recently described the cytotoxic effects of silica particles engulfed by macrophages. The presence of silica particles within phagosomes renders the membrane of the phagosome more permeable or actually ruptures it so that the released lysosomal enzymes enter the cytoplasm and may destroy the cell. In this way, activators of immune responses, whether they be modified antigens of enhanced immunogenicity or macrophage cytoplasmic RNA as suggested by Fishman, Hammerstrom and Bond (1963), may be released and stimulate immunologically competent cells. Transfer of information from macrophages to lymphocytes and plasma cells might in this way be accelerated by the action of silica and possibly also of substances such as bacterial endotoxin, on lysosomal and phagosomal membranes.

Normal guinea-pig serum contains 7S γ_1- and 7S γ_2-immunoglobulins as well as γM-globulin. After most methods of protein antigen injection, the guinea-pig responds principally by production of γ_1-antibody globulin. Only in exceptional circumstances is γ_2-immunoglobulin antibody produced against the new antigen. The addition of mycobacteria to Freund's adjuvant mixtures is essential for γ_2-globulin production and, as shown in the present communication, a similar effect sometimes occurs using silica adjuvants. It is not known why γ_1-antibody production can be stimulated so much more easily than γ_2-antibody production in the guinea-pig. The principal immunoglobulin in the serum of normal guinea-pigs is γ_2-immunoglobulin and it is therefore curious that by most methods of antigen administration γ_1-antibody results. Whereas in many species such as the rabbit, monkey, pig and man, antigenic stimulation leads principally to the appearance of antibody of the electrophoretic mobility of γ_2-globulin (Tiselius and Kabat, 1939), in other species such as the mouse and chicken, the main γ-globulin has a mobility which is intermediate between that of γ_1- or T-globulin in the horse (Richards and Marrack, 1963) and that of γG-globulin in man. Antigenic stimulation in these latter species leads to production of 7S antibody comparable in mobility to guinea-pig γ_1-globulin.

Using mycobacterial adjuvants a close correlation is found between γ_2-globulin antibody production and corneal delayed hypersensitivity. Such a convincing correlation cannot clearly be demonstrated using bentonite although the same association may be present. Boyden (1957) showed that prior injection of tuberculo-protein blocked the development of delayed hypersensitivity reactions after a subsequent immunization with BCG in

guinea-pigs. These guinea-pigs produced circulating antibodies against tuberculoprotein and Boyden suggested either that these antibodies neutralized the antigen and blocked the delayed reaction or that a secondary response involving production of circulating antibody interfered with the ability of the lymphoid cells to mount a delayed hypersensitivity reaction against these antigens.

As seen from Table 3 the previous injection of ovalbumin in water-in-oil emulsions (Group B) alters in several respects the subsequent response to an injection of the same antigen in complete Freund's adjuvant mixture, i.e. including mycobacterial peptidoglycolipid. Thus the granuloma at the site of injection is decreased in size at 2 weeks after injection and the ulceration of this lesion is obviously less severe. The latter observation indicates that delayed-type hypersensitivity may have been decreased in the group of previously injected animals (Group B). Group B animals also had a generally decreased precipitin arc of γ_2-immunoglobulin. The results of corneal tests in Group A animals were variable and no great decrease in corneal reactivity to ovalbumin was apparent in the previously injected group of animals (Group B).

A more clear-cut correlation between an induced decrease in delayed-type hypersensitivity and a decrease of γ_2-immunoglobulin has emerged from subsequent blocking experiments which were planned to be similar to the above but which used human serum albumin in the place of ovalbumin. As seen from Table 4, the group of previously injected animals (Group D) showed strikingly less corneal responses than the control Group C animals. This result therefore agrees with similar experiences which have been described by Asherson (1965) using the same antigen, human serum albumin.

These results with silica and mycobacterial adjuvant mixtures therefore suggest a linkage between the three events: macrophage–epithelioid granuloma production, delayed-type hypersensitivity and γ_2-immunoglobulin production in the guinea-pig, so that under a variety of experimental circumstances all three appear to vary in parallel. If delayed-type hypersensitivity can be shown eventually to depend for its occurrence upon production of an antibody globulin it may prove that the conditions required for the synthesis of the latter are the same as those required to induce an increased production of γ_2-immunoglobulin.

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REFERENCES

Allison, A. C., Harington, J., Birbeck, M. and Nash, T. (1965). 'Lysosomal enzymes in silica cytotoxity.' *Proceedings of the Second International Symposium on Inhaled Particles and Vapours*. Cambridge. (In press).

Asherson, G. L. (1965). 'Immune deviation: the inhibition of the characteristic effect of antigen in Freund's complete adjuvant by the prior injection of antigen.' *Biochem. J.*, **95**, 14P.

Askonas, B. A., White, R. G. and Wilkinson, P. C. (1965). 'Production of γ_1- and γ_2-antiovalbumin by various lymphoid tissues of the guinea pig.' *Immunochemistry*, **2**, 329.

Benacerraf, B., Ovary, Z., Bloch, K. J. and Franklin, E. C. (1963). 'Properties of guinea pig 7S antibodies. I. Electrophoretic separation of two types of guinea pig 7S antibodies.' *J. exp. Med.*, **117**, 937.

Boyden, S. V. (1957). 'The effect of previous injections of tuberculoprotein on the development of tuberculin sensitivity following BCG vaccination in guinea-pigs.' *Brit. J. exp. Path.*, **38**, 611.

Bozicevich, J., Bunim, J. J., Freund, J. and Ward, S. B. (1958). 'Bentonite flocculation test for rheumatoid arthritis.' *Proc. Soc. exp. Biol. (N.Y.)*, **97**, 180.

FISCHEL, E. E., KABAT, E. A., STOERK, H. C. and BEZER, A. E. (1952). 'The role of tubercle bacilli in adjuvant emulsions on antibody production to egg albumin.' *J. Immunol.*, **69**, 611.

FISHMAN, M., HAMMERSTROM, R. A. and BOND, V. P. (1963). 'In vitro transfer of macrophage RNA to lymph node cells.' *Nature (Lond.)*, **198**, 549.

MARTINDALE, W. (1952). *The Extra Pharmacopoeia*, 23rd edn, p. 155. The Pharmaceutical Press, London.

PERNIS, B. and PARONETTO, F. (1962). 'Adjuvant effect of silica (tridymite) on antibody production.' *Proc. Soc. exp. Biol. (N.Y.)*, **110**, 390.

RHODES, M. B., AZARI, P. R. and FEENEY, R. E. (1958). 'Analysis, fractionation and purification of egg-white proteins with cellulose cation exchanger.' *J. biol. Chem.*, **230**, 399.

RICHARDS, C. B. and MARRACK, J. A. (1963). 'γ-$_1$ Globulin: a low molecular-weight component of fowl serum.' *Protides biol. Fluids.* **11**, 144.

STEWART-TULL, D. E., WILKINSON, P. C. and WHITE, R. G. (1965). 'The affinity of a mycobacterial glycopeptide for guinea-pig gamma-globulin.' *Immunology*, **9**, 151.

SUTER, E. and WHITE, R. G. (1954). 'The response of the reticulo-endothelial system to the injection of the 'purified wax' and lipopolysaccharide of tubercle bacilli.' *Amer. Rev. Tuberc.*, **70**, 793.

TISELIUS, A. and KABAT, E. A. (1939). 'An electrotrophetic study of immune sera and purified antibody preparations.' *J. exp. Med.*, **69**, 119.

WHITE, R. G., COONS, A. H. and CONNOLLY, J. M. (1955). 'Studies on antibody production. IV. The role of wax fraction of *Mycobacterium tuberculosis* in adjuvant emulsions on the production of antibody to egg albumin.' *J. exp. Med.*, **102**, 83.

WHITE, R. G., JENKINS, G. C. and WILKINSON, P. C. (1963). 'The production of skin-sensitizing antibody in the guinea-pig.' *Int. Arch. Allergy*, **22**, 156.

WHITE, R. G., JOLLES, P., SAMOUR, D. and LEDERER, E. (1964). 'Correlation of adjuvant activity and chemical structure of wax D fractions of mycobacteria.' *Immunology*, **7**, 158.

WHITE, R. G. and MARSHALL, A. H. E. (1958). 'The role of various chemical fractions of *M. tuberculosis* and other mycobacteria in the production of allergic encephalomyelitis.' *Immunology*, **1**, 111.