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THE ROLE OF TUBERCLE BACILLI IN ADJUVANT EMULSIONS ON ANTIBODY PRODUCTION TO EGG ALBUMIN[1]

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For many years, the addition of a variety of substances including alum, tapioca, paraffin oil and staphylococcal toxoid to various antigens has been used to enhance antibody formation (for reviews cf. 1, 2). One of the adjuvant combinations most widely used in experimental work in recent years was developed by Freund and McDermott (3) following observations made by earlier investigators (cf. 1, 2). This procedure has come into widespread use with various antigens (cf. 1) and has been of especial value for the rapid production of acute disseminated encephalomyelitis in various species (4–9) using emulsions containing homologous or heterologous brain tissue. The relative importance of each of the ingredients has been evaluated (3, 10, 11, 12).

The contribution of the tubercle bacilli or other microorganisms, *Mycobacterium butyricum*, *Mycobacterium phlei*, *Nocardia asteroides* (13, 14, 15), to the potentiating effect of the Freund adjuvant mixture has been appraised in various systems. When the antigen-adjuvant emulsion was used without tubercle bacilli or the other potentiating microorganisms, lower titers of antibody or diminished immunity were found to horse serum (3), influenza PR8 virus (14), poliomyelitis virus (36) and *Plasmodium knowlesi* (16). On the other hand, addition of tubercle bacilli to an emulsion of typhoid bacilli, paraffin oil, water and aquaphor injected in rabbits had a slight potentiating effect on the typhoid agglutination titer (17, 37) and little or no effect on the antibody response to a similar emulsion of *Shigella paradysenteriae Flexner* (18). *Mycobacterium tuberculosis*, *Myco. butyricum* or *N. asteroides* were found to be essential in the emulsion mixture for the rapid production of disseminated encephalomyelitis (4, 6, 19) and for the production of complement fixing antibodies to normal brain (38). In the latter study (38), the possibility that the antibodies were antibodies to constituents of brain other than nervous tissue was not considered (cf. 39). Injection of emulsions containing dead tubercle bacilli at sites other than those containing brain emulsion without tubercle bacilli failed to produce disseminated encephalomyelitis (19).

In addition to enhanced antibody response resulting from materials in particulate form as compared with those in solution and from the delayed absorption of

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antigen from a focus, many investigators have commented on the unique potentiating effect on antibody formation of certain types of inflammatory reaction such as that produced by the tubercle bacillus and other microorganisms (2, 11, 20, 21) and Freund, Casals and Genghof (11) suggested that the epithelioid cells in the local granuloma formed to tubercle bacilli in paraffin oil might be responsible for the enhanced antibody formation to this microorganism.

It has been hypothesized that the mechanism of the production of the acute disseminated encephalomyelitis involved the local formation of antibody at the sites of the brain-adjuvant emulsion (15, 19, 22). The tubercle bacilli are considered to function by attracting to the site of the brain material a high concentration of epithelioid cells (1, 11, 15, 19, 22) which are thought to produce the antibody. To obtain further data in relation to this hypothesis, it was considered of interest to study by quantitative immunochemical methods the antibody response in guinea pigs receiving Freund-type emulsions of crystalline egg-albumin with and without killed tubercle bacilli, and to correlate the findings with the histological study of the inoculation sites, regional lymph nodes and spleen.

The findings indicate that a substantially increased and prolonged antibody response to emulsions of crystalline egg-albumin containing killed tubercle bacilli was obtained as compared with the antibody response to similar emulsions lacking tubercle bacilli and that the enhanced antibody response could be correlated with the presence of a local granuloma at the sites of inoculation. In addition marked hyperplasia of the regional lymph nodes and moderate splenic hyperplasia with an unusual splenic lesion occurred in animals which received emulsions containing tubercle bacilli.

EXPERIMENTAL

Four times recrystallized egg albumin was used as antigen. Two preparations of antigen-adjuvant emulsion were made by mixing in a mortar the ingredients in the following proportions: 500 mg egg-albumin dissolved in 25 ml saline, 25 ml aquaphor and 50 ml of paraffin oil. To one mixture, 64 mg of dried tubercle bacilli (strain H 37 RV) were added; these were ground and incorporated into the paraffin oil before the oil was added to the other ingredients. The other mixture contained the same materials except for the tubercle bacilli.

Guinea pigs weighing between 400–600 g were distributed according to sex and weight in an attempt to achieve two groups comparable in these respects. One group (T) of 28 guinea pigs received the emulsion containing tubercle bacilli and the other group (A) of 15 guinea pigs received the emulsion without tubercle bacilli.

Three injections of 1 ml of the antigen-adjuvant mixtures were given intramuscularly at weekly intervals at different sites in the following order: right shoulder girdle, left shoulder girdle and mid-sacral area. Animals were bled by cardiac puncture at 2, 4, 6 and 8 weeks after the third and last injection of antigen-adjuvant emulsion. In the third week after the last injection, intracutaneous tuberculin tests with second strength PPD were done in a few of the animals in each group. Those in group T which had received killed tubercle bacilli gave

strongly positive tuberculin reactions, while negative reactions were obtained in the animals of group A. After the second bleeding some of the animals in group T were transferred to a concurrent experiment and data on them are not included after that period.

Sera were allowed to remain in the refrigerator at 4 C until hemolytic complement activity had disappeared. Analyses for anti-egg-albumin nitrogen were carried out in duplicate by the quantitative precipitin methods of Heidelberger and Kendall (23) with the Heidelberger and MacPherson (24) modification employing the Markham apparatus (25), since only small samples of blood were obtained. Based on preliminary precipitin tests (25), analyses were carried out on 0.5 ml or 1.0 ml samples of serum and the amount of antigen to be used in the quantitative precipitin tests was such as to give maximum precipitation of antibody and a slight excess of antigen in the supernate. When supernatant tests or the ratio of antibody N to antigen N in the precipitate indicated that this was not the case, analyses were repeated, when possible, with a more suitable quantity of antigen. Results in which maximum precipitation of antibody was not determined precisely are indicated in Table I. Antibody nitrogen was computed by subtracting the antigen nitrogen used. Sera are recorded as containing no antibody when 2 μg of egg-albumin nitrogen added to the sample of serum showed no evidence of precipitation on analysis after a week in the refrigerator. Such sera may have contained minimal amounts (less than 10 to 15 μg Ab N/ml) since occasional samples of 2.0 ml were able to sensitize guinea pigs passively so that fatal anaphylaxis (26) resulted on injection of antigen, while 1 ml samples did not.

RESULTS

The data in Table I show that inclusion of killed tubercle bacilli in the adjuvant emulsion produced in group T a dramatic increase in the antibody levels 2 weeks after the third injection as compared with the levels in group A the animals of which were injected with adjuvant emulsion lacking tubercle bacilli. In group T the median value was 116 to 122 μg of antibody nitrogen per ml (μg Ab N/ml) as compared with a median value in group A of 22 μg Ab N/ml. Furthermore, samples at 4, 6 and 8 weeks show that the animals in group T had a definitely prolonged antibody response as compared with those in group A. Indeed, many of the animals in group T showed substantial antibody levels at the end of 8 weeks while only one of those in group A had any demonstrable precipitin at the end of 8 weeks and only two had demonstrable precipitin at the end of 6 weeks.

Detailed descriptions and illustrations of local inoculation sites in animals receiving various antigens in Freund adjuvants with and without tubercle bacilli have been published (20, 21, 28). The present findings did not differ in any respect from earlier descriptions. Similarly, changes in the regional lymph nodes were like those repeatedly observed in hyperimmunized animals with (20, 21, 28) and without (27) adjuvants and in a variety of chronic infections (27).

An unusual histological finding was found in the spleen which does not appear to have been previously described. While the spleens of animals in Group A were

TABLE I

Antibody response of guinea pigs immunized to crystalline egg albumin in emulsion with adjuvants containing and lacking killed tubercle bacilli

WEEKS AFTER THIRD INJECTION			
2	4	6	8
Serum antibody levels			
μg AbN/ml	μg AbN/ml	μg AbN/ml	μg AbN/ml
Group T—with tubercle bacilli			
0	—	—	—
3	—	—	—
22	1	0	0
47	89	0	0
60	54		
68	139	208	90
76	100		
83	—	—	—
89	92	55	39
95	73	28	24
102†	152	144	117
104	113	67	18
108	122		
116*	163	137	48
122*	89	—	—
122	144		
125	121	—	—
125	143	130	79
129	106	248	—
145	34	15	0
148	155		
167	142		
184	165	151	94
190	—	—	—
196	199		
207	192	—	—
212	252	196	84
214‡	—	—	—
Group A—without tubercle bacilli			
0	—	—	—
0	0	—	—
0	0	0	0
0	0	0	0
6	—	—	—
7	3	0	0
11	0	—	—
22*	9	0	0
27	0	0	0
28	—	—	—
30	0	0	0
46	0	0	0
71	28	—	—
108	—	21	0
170	60	34	10

* Indicates median values at second week.

† Analysis may be low due to excessive antigen.

‡ Analysis may be low since antibody was detectable in supernatant.

not enlarged and exhibited no abnormalities (Fig. 1), those of Group T which had received tubercle bacilli, in addition to gross enlargement, showed hyper-

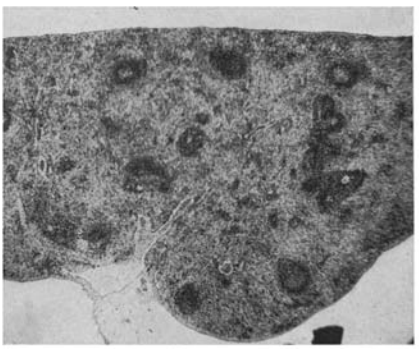

FIG. 1

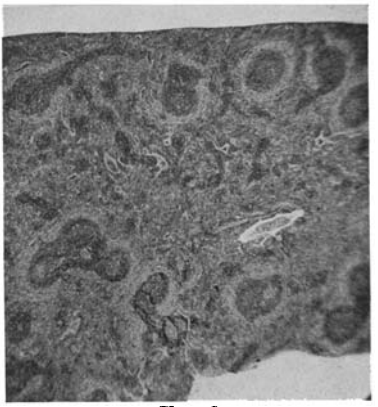

FIG. 2

FIG. 1. Spleen of guinea pig of group A. ×50

FIG. 2. Spleen of guinea pig of group T. Enlarged follicles are surrounded by halo of fibrous tissue. ×50.

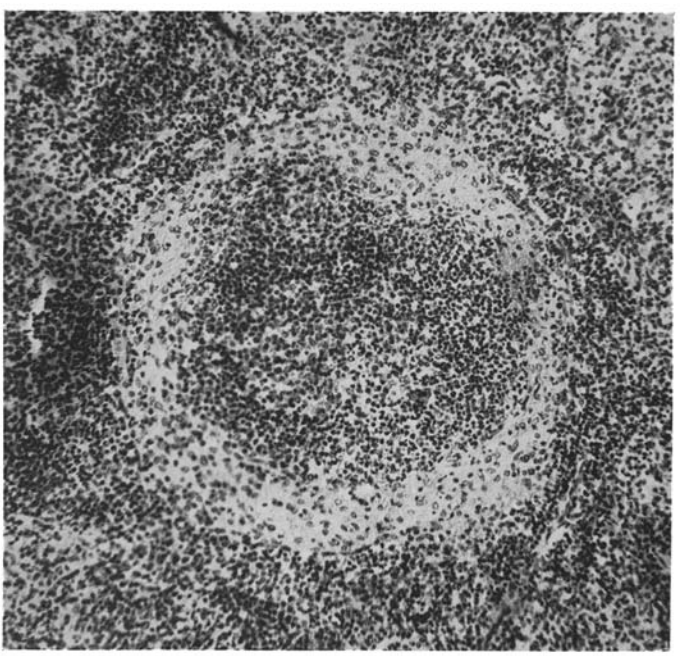

FIG. 3. Higher power of isolated Malpighian corpuscle from previous section. H. & E. ×120.

plastic changes (Fig. 2) similar to those seen in the lymph nodes (cf. 20, 21, 28) but no granulation tissue except for an isolated instance. In the majority of these

spleens, the Malpighian corpuscles were surrounded by a rather heavy rim of fibrous tissue arranged in concentric lamellae with some crossing over of individual fibers (Figs. 3 and 4). These areas of perifollicular fibrosis were devoid of fibrin or argyrophilic reticulum (Fig. 4).

No appreciable differences were discernible at the different sites of injection or in animals dying during the course of the experiment as compared with those sacrificed at the end of the experiment so that the duration of the existence of the lesions at the local inoculation sites did not appreciably alter their morphologic appearance.

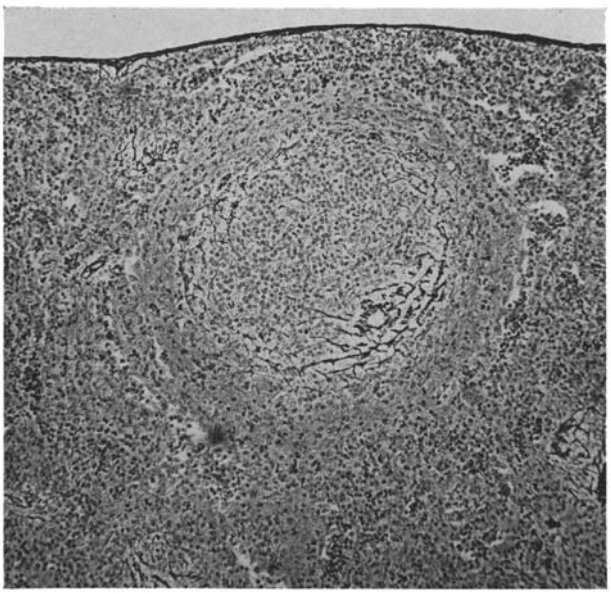

FIG. 4. Similar structure after silver impregnation (Wilder) demonstrating the lack of reticulum fibers in the fibrotic areas. ×120.

DISCUSSION

This study has confirmed and measured in quantitative terms the augmentation and prolongation of the antibody response attributable to the presence of killed tubercle bacilli in the Freund antigen-adjuvant emulsion. Previous reports have indicated the relative importance of killed tubercle bacilli in various systems (4, 6, 12–16, 19, 36, 38), but heretofore the degree of potentiation has not been measured in absolute terms. There is a fivefold increase in amount of antibody nitrogen at the median in animals in group T as compared with that of group A 2 weeks after the last injection of antigen and an even more striking difference is noted at a later period. The more prolonged antibody levels in the animals of group T are also evident at 4, 6 and 8 weeks after the last antigen-adjuvant injection.

The differences in amount and duration of antibody response to crystalline egg albumin found in this study are attributable solely to the addition of killed

tubercle bacilli. These organisms probably exert their effect by eliciting an inflammatory reaction at the local site, in the regional lymph nodes and elsewhere as has been hypothesized (11, 15, 19, 22). The details of the manner in which such changes result in the increased antibody formation remains unknown (cf. 1).

Without tubercle bacilli, the injection of crystalline egg albumin, saline, aquaphor and paraffin oil results in an innocuous appearing foreign body reaction. There is no evidence of reaction in the regional lymph nodes or spleen. With killed tubercle bacilli in the antigen-adjuvant mixture, a cellular granuloma formed at the site of injection and a similar type of reaction is seen in the regional lymph nodes. The spleens of these animals showed enlargement and hyperplasia of follicles (20, 21, 28). It has been reported that the lungs also show similar granulomatous changes (1, 20, 21). These changes may contribute not only to the delayed absorption of antigen and to its intimate association with cells which form antibody at the site of injection, but also may serve to direct the antigen toward lymph nodes and other sites in the body that are associated with antibody production (20, 21, cf. 1).

In addition to the evidence for the production of antibody in certain organs (for a review cf. 29), there is considerable evidence that formation of antibody may occur in relatively localized areas (30) and especially in granulomas produced by various antigen-adjuvant combinations (31–34). Early excision of an antigenic depot of paraffin oil and tubercle bacilli resulted in diminished antibody production (32, cf. 1) and in several instances Westwater (32) found complement fixing antibody in extracts of granuloma before it could be detected in the blood. Regional lymph nodes draining such granulomata were also found to contain antibody (35) but Ehrich, Harris and Mertens (35) were unable to detect it in extracts of the granulomas, possibly because of the presence of excess antigen.

SUMMARY

Guinea pigs injected with crystalline egg albumin in an emulsion with the Freund adjuvants and containing killed tubercle bacilli showed substantially increased and prolonged antibody levels in their serum measured by the quantitative precipitin reaction as compared with guinea pigs injected with a similar emulsion lacking killed tubercle bacilli. The increased antibody formation was correlated with local granuloma formation and hyperplasia in the regional lymph nodes and spleens of animals receiving the emulsion containing killed tubercle bacilli.

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