Discussion. ThioTEPA-induced hyperpigmentation of the extremities, tail, and ears of brown or black mice with sparing of the general body skin is similar to that described following total-body irradiation(1), both agents resulting in increased deposition of epidermal melanin and increased numbers of epidermal and dermal melanocytes. Presumably the greater number of melanotic melanocytes present in the extremities, tail, and ears(4) and the slower conversion of amelanotic to melanotic melanocytes in the general body skin(1) account for these findings. Szabó (5) has suggested that melanocytes of hair follicles can be stimulated with dimethylbenzanthracene and croton oil to function outside of their original environment. No significant increase in hair follicle melanocyte activity was detected in treated mice. The present study indicates, in addition, that similar microscopic changes occur to a lesser degree in mice of lighter coat color, but not in albinos. Further studies will determine whether ThioTEPA given in larger doses or for a longer time can cause gross hyperpigmentation even in these lighter coat-colored strains. The possible role of the adrenal or gonads in ThioTEPA-induced pigment changes is not clarified by these findings. All adrenals appeared normal histologically. All males and most females treated were apparently rendered sterile on the basis of histologic observations. It has been shown that ovariectomy will interfere with melanogenic activity(6).

Summary. Systemic administration of ThioTEPA caused the development of hyperpigmentation of the ears, tail, and extremities of brown or black mice, but not of mice of lighter coat color or albinos. These changes resulted from increased numbers of dermal and epidermal melanocytes and increased deposition of epidermal melanin. Similar microscopic changes were seen to a lesser degree in the lighter-colored mice, but not in albinos. Gross or microscopic evidence of hyperpigmentation of the general body skin was not found.

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Effect of Mycobacteria on Antibody Production Induced by Water-in-Oil Emulsions of Soluble Protein Antigens.* (26168)

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It has been well established that the sensitizing potency of tissue antigens in water-in-oil emulsions is augmented when mycobacteria are added. Thus, hypersensitivity states such as allergic encephalomyelitis, aspermatogenesis and thyroiditis are more readily produced when appropriate tissue antigens are incorporated into adjuvants containing mycobacteria than when the mycobacteria are omitted. On the other hand, the effect of mycobacteria on ability of antigens in emulsions to stimulate antibody formation is not clear. Addition of mycobacteria to water-in-oil emulsions containing viral, bacterial and

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rickettsial antigens has been reported to enhance greatly(1-3), to enhance slightly(4-5), or to have no enhancing effect(6-9) on production of circulating antibody. Also, numerous investigators have reported that mycobacteria or wax from mycobacteria greatly enhanced production of circulating antibody in animals injected with water-in-oil emulsions of soluble protein antigens(10-14), whereas others have reported that mycobacteria had little or no effect on antibody production in animals injected with emulsions of these same antigens(15-17). In preliminary studies in this laboratory with partially purified protein antigens, it was observed that mycobacteria enhanced production of circulating antibody in guinea pigs with one immunization scheme but not with another. The present investigation was initiated to determine the effect of mycobacteria on antibody production elicited by different immunization schemes.

Materials and methods. Protein antigens were obtained from the following sources: bovine gamma globulin (BGG), Armour and Co., Lot No. C-906 and C-904; and egg albumin (Ea), Armour and Co., Lot No. E-9015. BGG was trace labelled with I^{131} by the method previously described(18).

Adjuvants‡ were prepared with 9 parts bayol F (ESSO Standard Oil Co.), 1 part Arlacel C (Atlas Powder Co., Wilmington, Del.), and 10 parts of 0.15 M NaCl solution containing the protein antigen. The oil and emulsifier were first mixed in a homogenizer, then the protein solution was added and the mixture homogenized at 0-3°C until a paste-like emulsion was obtained. A similar adjuvant containing Arlacel A instead of Arlacel C was employed by Salk and Laurent(19). When mycobacteria (*M. tuberculosis hominis*) were incorporated into the adjuvant, they were mixed into the oil before addition of the emulsifier and protein solution. Male guinea pigs weighing 450-500 g were injected with a total of 15 mg of either BGG or Ea in adjuvant, with or without mycobacteria. Three different methods of injecting and bleeding were used. The first method was similar to that reported by Fischel *et al.*(12). Guinea pigs were injected once a week for 3 consecutive weeks with 1 ml of adjuvant containing 5 mg of antigen per ml. Half of the animals received adjuvant containing 0.64 mg of mycobacteria/ml, and half received adjuvant containing no mycobacteria. Each injection was given subcutaneously in multiple sites, including 0.1 ml in each foot pad. The guinea pigs were bled by cardiac puncture, 13 days, 4 weeks, and 7 weeks after last injection, and the sera were analyzed for precipitating antibody(20). The third method was similar to that described by White *et al.* (13). A single hind foot pad (subcutaneous) injection of 0.2 ml of adjuvant containing 15 mg of the antigen was given to each guinea pig. Half of the guinea pigs received adjuvant containing 1 mg mycobacteria and the other half received adjuvant containing no mycobacteria. The animals were bled 3 and 7 weeks after injection and the sera were analyzed for precipitating antibody. Guinea pigs were also injected in the same manner with adjuvant containing I*BGG with or without mycobacteria, and the disappearance of the radioactivity from both the whole animals and the foot pads was followed. The I* activity in both the entire animals and injection site (feet and lower hind legs) of these guinea pigs was determined periodically in a gamma well-type counter beginning on the first day following injection. Counts in the uninjected legs were less than 1% of the counts in the injected leg and were subtracted as non-specific background from the counts in the injected legs.

Results. High levels of precipitating antibody were produced in guinea pigs which received 3 weekly injections of BGG in adjuvant (3.0 ml total), both with and without mycobacteria (Table I). There was no significant difference in mean antibody concentration of the sera obtained from the 2 groups of animals during the period of study. However, 7 weeks after injection, the group receiving adjuvant without mycobacteria weighed 25% more than the group which received mycobacteria. Both those animals receiving mycobacteria and those not receiving

‡ The term "adjuvant" will be used throughout this paper to denote a water-in-oil emulsion containing antigen.

TABLE I. Antibody Production in Guinea Pigs Receiving Multiple Injections of BGG in Adjuvant.

Guinea pig No.	μg antibody N/ml serum 13 days	4 wk	7 wk
	(Without mycobacteria)		
1	532.0	592.5	—
2	768.0	—	—
3	652.0	—	—
4	506.0	684.5	729.5
5	493.0	796.0	748.0
6	642.0	695.0	722.0
7	681.0	—	—
8	507.0	734.5	635.5
9	599.0	977.5	836.5
10	296.0	420 5	—
11	960.0	927.0	792.0
12	407.0	418.0	312.0
13	820.0	844.5	771.5
14	569.0	771.0	710.0
15	535.0	—	—
Avg Ab. N/ml	597.8	714.6	695.2
Avg wt (g)	641	724	799
	(With mycobacteria)		
1	473.0	597.0	567.5
2	258.0	273.0	—
3	297.0	603.5	524.5
4	439.0	629.5	608.5
5	741.5	696.0	—
6	725.0	—	—
7	706.0	582.0	—
8	690.5	683.5	—
9	745.0	770.0	774.0
10	568.0	724.0	718.0
11	559.0	834.5	741.0
12	377.5	595.0	580.5
13	613.5	752.0	749.0
14	404.0	578.0	—
15	604.0	—	—
Avg Ab. N/ml	546.7	639.8	657.9
Avg wt (g)	501	506	598

mycobacteria maintained high levels of antibody in their sera during the 7 week period. In fact, 7 weeks after last injection, there was more antibody present in the sera than 13 days following last injection. Similar results were obtained in guinea pigs which received 3 weekly injections of Ea in a total of 3 ml of adjuvant (Table II). In these animals, mycobacteria had a slight augmenting effect on concentration of precipitating antibody which was most noticeable soon after injections were terminated. However, the group of animals injected with mycobacteria did not gain weight as rapidly as did the group not injected with mycobacteria, so that total amount of antibody circulating per animal was somewhat greater in the animals receiving adjuvant without mycobacteria.

In contrast to the above results, mycobacteria had a marked enhancing effect on antibody production in guinea pigs given a single injection of BGG or Ea in 0.2 ml of adjuvant (Tables III and IV). Although the guinea pigs which did not receive mycobacteria gained somewhat more weight during the post injection period, this could account for only a small part of the difference in antibody levels.

A lesser augmenting effect of mycobacteria on antibody production was observed in guinea pigs which received a single injection of 3.0 ml of adjuvant (Tables V and VI). Antibody levels in sera of the group which received no mycobacteria were 35% (BGG) and 32% (Ea) lower at 7 weeks than those

TABLE II. Antibody Production in Guinea Pigs Receiving Multiple Injections of Egg Albumin in Adjuvant.

Guinea pig No.	μg antibody N/ml serum 13 days	4 wk	7 wk
	(Without mycobacteria)		
1	82.2	81.6	74.6
2	341.2	376.0	402.2
3	182.2	259.6	296.4
4	270.4	—	—
5	128.4	116.8	109.2
6	192.2	240.0	222.4
7	381.6	470.0	457.2
8	240.4	267.2	364.0
9	350.0	356.4	362.4
10	401.6	442.4	464.8
11	385.6	—	—
12	175.2	163.6	233.2
13	302.0	326.0	—
14	87.6	103.8	111.6
Avg Ab. N/ml	251.5	266.7	281.5
Avg wt (g)*	625	689	764
	(With mycobacteria)		
1	448.0	496.0	485.6
2	125.2	96.4	123.8
3	447.2	487.0	—
4	385.2	—	—
5	354.0	318.0	422.8
6	97.8	130.2	135.2
7	102.6	121.4	119.2
8	354.8	448.0	325.2
9	362.8	286.8	222.4
10	291.6	290.4	—
11	499.6	—	—
12	496.4	520.0	667.6
13	446.8	—	—
Avg Ab. N/ml	339.4	319.4	312.7
Avg wt (g)†	471	465	511

* Avg wt before inj. = 482.
† *Idem* = 496.

TABLE III. Antibody Production in Guinea Pigs Receiving a Single Injection of BGG in 0.2 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk
(Without mycobacteria)			(With mycobacteria)		
1	0	18.0	1	242.0	359.2
2	0	5.8	2	118.0	147.2
3	0	—	3	95.2	302.4
4	0	25.8	4	88.2	213.6
5	0	0	5	73.6	70.0
6	0	18.4	6	63.2	133.6
7	19.8	—	7	56.0	78.4
8	0	10.8	8	30.8	26.8
Avg Ab. N /ml serum		13.1		95.9	166.4
Avg wt, g*	545	708		481	606

* Wt at time of inj.: without mycobacteria, 472 g; with mycobacteria, 475 g.

in sera of the group which received mycobacteria. However, a large portion of this difference is offset by a difference in the weights of the animals. The groups which received no mycobacteria weighed 21% (BGG) and 22% (Ea) more at 7 weeks post injection than the groups receiving mycobacteria. Assuming a similar plasma protein pool to body weight ratio for both groups, there was only 21% (BGG) and 17% (Ea) less antibody produced in guinea pigs not receiving mycobacteria than in those receiving mycobacteria with this immunization scheme.

TABLE IV. Antibody Production in Guinea Pigs Receiving a Single Injection of Ea in 0.2 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk
(Without mycobacteria)			(With mycobacteria)		
1	102.4	49.6	1	124.0	
2	81.6	261.2	2	307.2	
3	7.6	20.4	3	349.6	244.0
4	104.4	202.8	4	398.4	324.2
5	74.0	180.8	5	372.6	—
6	26.6	—	6	416.4	—
7	29.2	52.0	7	391.6	430.4
8	54.2	—	8	255.2	362.0
9	24.2	40.6	9	359.2	—
10	15.4	47.0	10	342.4	369.6
11	10.8	11.8	11	147.6	224.0
Avg Ab. N /ml serum	48.2	96.2		306.3	325.7
Avg wt, g*	512	712		497	627

* Wt at time of inj.: without mycobacteria, 445 g; with mycobacteria, 447 g.

I*BGG in adjuvant injected into foot pads of guinea pigs disappeared somewhat more slowly when mycobacteria were incorporated into the adjuvant than when they were excluded (Table VII). A difference was not apparent until approximately 10 days after injection. The difference increased with time until, after 21 days, the injection sites in the

TABLE V. Antibody Production in Guinea Pigs Receiving a Single Injection of 15 mg BGG in 3.0 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk
(Without mycobacteria)			(With mycobacteria)		
1	57.2	212.8	11	335.2	468.6
2	254.4	320.4	12	135.2	232.8
3	207.6	182.8	13	426.6	374.8
4	150.8	242.4	14	341.6	—
5	63.4	101.6	15	147.6	208.8
6	231.2	224.4	16	396.6	—
7	226.8	—	17	345.4	472.2
8	267.6	—	18	362.6	333.6
9	56.4	104.8	19	422.2	—
10	63.0	364.8	20	427.0	358.0
Avg Ab. N /ml	157.8	219.3		334.0	349.8
Avg wt, g*	574			515	

* Wt at time of inj.: without mycobacteria, 461 g; with mycobacteria, 467 g.

TABLE VI. Antibody Production in Guinea Pigs Receiving a Single Injection of 15 mg Ea in 3.0 ml of Adjuvant.

Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk	Guinea pig No.	μg antibody N/ml serum 3 wk	7 wk
(Without mycobacteria)			(With mycobacteria)		
21	197.4	249.8	31	556.8	546.7
22	286.2	220.2	32	324.0	—
23	153.4	187.4	33	556.8	487.1
24	105.7	—	34	592.4	515.6
25	277.2	—	35	323.6	462.0
26	357.6	509.2	36	600.8	534.4
27	534.4	531.2	37	638.0	—
29	312.8	299.2	38	214.2	361.6
30	199.8	292.2	39	266.6	—
			40	280.4	446.0
Avg Ab. N /ml	266.1	327.0		435.4	479.1
Avg wt, g*	588			490	

* Wt at time of inj.: without mycobacteria, 458 g; with mycobacteria, 465 g.

TABLE VII. Per Cent† of I*BGG Remaining in Injection Sites of Guinea Pigs.

Material inj.	No. of guinea pigs	Days following inj. 1	3	6	8	10	13	17	21	μg antibody N per ml serum 3 wk	7 wk
15 mg I*BGG in adjuvant											
Without mycobacteria	6	73.7	46.3	28.1	19.9	15.1	10.8	7.7	3.9	5.9	21.4
With mycobacteria	6	68.2	46.5	31.4	20.2	17.1	12.6	10.5	7.0	77.3	174.1

Guinea pigs were inj. in left hind foot pad with 0.2 ml of adjuvant.
† % of total I* activity inj.

guinea pigs which received mycobacteria possessed nearly 100% more I* activity than the group receiving no mycobacteria. At this time, the group which received the mycobacteria also had much higher levels of precipitating antibody. In another experiment in which I* activity was also determined in the whole body, a greater amount of I* activity was present after the tenth day in both the injection site and the whole body of guinea pigs receiving mycobacteria than in injection site and whole body of guinea pigs receiving no mycobacteria (Table VIII).

The granulomata developing around the depots of adjuvant with mycobacteria were consistently much larger than those developing about adjuvant without mycobacteria. This was true even when sites receiving only 0.2 ml adjuvant plus mycobacteria were compared with sites receiving 0.5 ml adjuvant without mycobacteria.

Initially, the histologic reactions to the adjuvant without mycobacteria, either in injection depot or in distant lymphatic tissues, were made up of a moderate number of lipophages, lymphocytes, a few plasma cells and occasional polymorphonuclear leukocytes. Within the next few weeks the number of lymphocytes and plasma cells about the lipid droplets increased and fibroblasts proliferated, in part walling off the droplets. During the first several weeks following injection, the number of large lipophages decreased. The granulomas that developed about the adjuvant with mycobacteria in the local depot or distant lymphoid tissues were conspicuous and consisted of great numbers of large mononuclear cells, some containing visible droplets of oil and others having a granular eosinophilic cytoplasm. Many giant cells were also present. These appeared to have been formed by coalescence of several large mononuclear cells. Moderate numbers of polymorphonuclear leukocytes, lymphocytes and plasma cells were also scattered through the granulomas. These granulomatous reactions were extensive, involving and frequently destroying adjacent striated muscle fibers. During the first several post injection weeks, some fibrosis took place about the droplets and occasional abscesses developed; otherwise the extensive granulomata remained histologically unchanged.

TABLE VIII. Per Cent of I*BGG Remaining in Guinea Pigs.

No. of guinea pigs	Area counted	Days following inj.† 3	5	7	8	10	14	16
	Without mycobacteria							
6	Foot	74	52	37	32	25	12	11
	Whole body‡	72	53	34	34	26	14	13
	With mycobacteria							
6	Foot	78	51	38	33	28	19	17
	Whole body‡	75	49	42	35	26	19	18

Guinea pigs were inj. in left hind foot pad with 0.2 ml of adjuvant containing 15 mg I*BGG.
† % of I* activity present on day 1.
‡ Minimal values, since efficiency of detecting isotopes throughout body is slightly less than detecting isotopes in localized area.

Discussion. The effect of mycobacteria in water-in-oil emulsions on production of precipitating antibody to a constant amount of protein antigen in guinea pigs depends on the immunization scheme employed. Mycobacteria had no significant augmenting effect on antibody production to either BGG or Ea when multiple injections totaling 3.0 ml of adjuvant were given over a 3 week period. When 3.0 ml of the adjuvant were given in a single injection to guinea pigs, 30-35% more antibody was obtained per ml of sera when mycobacteria were used than when they were

not. However, when the antibody levels were calculated on a per animal basis, this difference was only 17-21%. On the other hand, mycobacteria had a marked enhancing effect on antibody production in guinea pigs injected with a single dose of 0.2 ml of adjuvant containing either BGG or Ea as previously demonstrated by White *et al.*(13). The amount of antibody produced following a single injection of 3.0 ml of adjuvant without mycobacteria was about the same as that produced after a single injection of 0.2 ml of adjuvant with mycobacteria. Since the same amount of antigen was given in both cases, it appears that in this instance a large volume of adjuvant lacking mycobacteria had as great an enhancing effect as did a small volume of adjuvant containing mycobacteria.

Perhaps the effectiveness of the antigen in adjuvant preparations determines the extent to which addition of mycobacteria will further enhance antibody production. In situations where the effectiveness in emulsion is great (3 ml of adjuvant in multiple injections), addition of mycobacteria to the adjuvant may not further enhance antibody production, but in situations where the effectiveness of the same amount of antigen is low (0.2 ml of adjuvant in a single injection), addition of mycobacteria may greatly enhance antibody production. The effectiveness of an antigen in an adjuvant preparation would be influenced by the immunization scheme employed, the species into which the adjuvant is injected, the antigen employed, and the physical state of the emulsion. Fischel *et al.*(12) observed a marked enhancing effect of mycobacteria on production of anti Ea in guinea pigs receiving multiple injections of Ea in adjuvant. The immunization scheme (amounts of antigen and mycobacteria injected, volume of adjuvant injected, etc.) was identical to one of the schemes (Tables I and II) used in the present study. The oil (paraffin) and emulsifying agent (aquaphor) differed from our oil and emulsifier and were mixed in different proportions. The adjuvant effect of the emulsion used by Fischel *et al.* was apparently inferior to that of the emulsion used in the present study, since the level of antibody produced in the present study without mycobacteria was approximately 3 times as high as that obtained by Fischel *et al.* with mycobacteria. Although all of the conflicting results published on the effect of mycobacteria on antibody production to antigen in adjuvant probably cannot be explained by a difference in the properties of the adjuvants employed, they may be explained by some other differences in the experimental situation which affect the effectiveness of antigen in adjuvant preparations; *e.g.*, animal species, volume of adjuvant injected, amount of antigen used, route of injection, or immunization scheme employed.

Adjuvants both with and without mycobacteria elicited a prolonged production of antibody. Antibody levels were generally higher 7 weeks following last injection than 2 to 4 weeks following last injection. A persistence of antibody production was observed in all of the animals in the present study and was independent of immunization scheme employed or level of antibody produced. This agrees with the work of Freund and Bonanto (21), who demonstrated agglutinating antibody in rabbits 3 years after injection of typhoid bacilli in adjuvant which either contained or lacked mycobacteria. On the other hand, Fischel *et al.*(12) reported that the precipitating anti Ea did not persist in sera of guinea pigs injected with adjuvant unless mycobacteria were added to the adjuvant. The physical state of the adjuvant employed might explain the failure of Fischel and coworkers to obtain prolonged antibody production without addition of mycobacteria.

There is no relationship between the size of granuloma produced at the injection site of adjuvant and antibody production. This is not surprising in view of the findings of White *et al.*(13), that only a small number of antibody containing cells in the local granuloma of guinea pigs injected with Ea in adjuvant either containing or lacking mycobacteria. Also, Askonas and White(22) demonstrated that the local granuloma produced by injection of adjuvant containing mycobacterium wax was relatively inactive in the *in vitro* synthesis of antibody. Apparently, mycobacteria do not serve to bring antibody producing cells to the local depot of adjuvant.

Our observations indicate a correlation between persistence of antigen and antibody formation, suggesting that one way in which mycobacteria might augment antibody response would be in increasing the retention of antigen. In the guinea pig, antigen was apparently eliminated more slowly from adjuvant with mycobacteria than from adjuvant without mycobacteria, and the former stimulated more antibody production. As might be expected, however, there is not a close correlation between amount of antigen retained and amount of antibody produced; mycobacteria and 0.2 ml of adjuvant elicited 12 times as much antibody in guinea pigs as did 0.2 ml of adjuvant alone, while mycobacteria and adjuvant only increased antigen retention by a factor of 2 over adjuvant alone. These data, while inconclusive, are consistent with the idea that mycobacteria in adjuvants enhance antibody production by increasing retention of the antigen.

Summary. In guinea pigs the effect of mycobacteria in adjuvant on production of precipitating antibody to a constant amount of antigen depends upon the immunization scheme employed. With multiple large injections of adjuvant, mycobacteria had no effect on antibody formation; with a single large injection, mycobacteria had a slight enhancing effect; and, with a single small injection, mycobacteria had an extreme enhancing effect. Mycobacteria had no effect on persistence of antibody production. There was a persistence of antibody production independent of the level of the antibody, with all the immunization schemes employed. Adjuvants containing mycobacteria produced considerably more extensive granulomata at injection sites than adjuvants lacking mycobacteria. However, there was no relationship between size of the granuloma produced at the injection site and antibody production. In guinea pigs injected with a small dose of adjuvant containing mycobacteria, there was both a greater persistence of antigen in the local adjuvant depot and in the whole body and a larger antibody production than when mycobacteria were omitted.

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