CRITICAL RELATIONSHIPS BETWEEN CONSTITUENTS OF THE ANTIGEN-ADJUVANT EMULSION AFFECTING EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN A COMPLETELY SUSCEPTIBLE MOUSE GENOTYPE*

By JOHANNA M. LEE, Ph.D., and HOWARD A. SCHNEIDER, Ph.D.

(*From The Rockefeller Institute*)

Plates 20 and 21

(Received for publication, August 22, 1961)

It seems fair to say that our understanding of such an incompletely understood disease as allergic encephalomyelitis is roughly proportional to the degree in which we approach certitude in our attempt to produce the disease and affect its incidence. The time is past for self-congratulation on being able to produce the disease erratically and with unpredictable frequencies. It seems permissible now to ask, as a next level of our understanding, that we begin to make some finer quantitative distinctions. In what follows we shall confine our demands, and our experiments, to a single host species, the mouse, and it will be our purpose to report some new operational discriminations in the specifications leading to the production of experimental allergic encephalomyelitis (EAE).[1]

The Reference Point

Any attempt to refine our discriminations in specifying the things we must do to produce EAE must begin with some reference point from which we can measure the consequences of manipulation. In the mouse model we have chosen as this reference point the 100 per cent incidence level based on histopathologic diagnosis. The mouse model uniquely offers us this choice at the present, for reasons which will become evident below. In the guinea pig, on the other hand, Kies and Alvord have constructed a "disease index" (2) following a similar impulse to devise means permitting discriminatory distinctions in the production of the disease. Their employment of clinical signs and clinico-chemical data in the "disease index" is not practicable in

* This investigation was supported in part by a research grant, B-1565(C), from the National Institute of Neurological Diseases and Blindness, Public Health Service. A preliminary report was published in *Fed. Proc.*, 1961, **20**, (Part 1), 263.

[1] In previous publications we have alluded to this disease as "acute disseminated encephalomyelitis," or ADE. There is much to recommend this terminology, based as it is on the pathologic description. Since the publication of the Kies and Alvord book "Allergic Encephalomyelitis" (1), there has been an increasing trend toward the use of "experimental allergic encephalomyelitis," or EAE, as a rubric for the phenomenon.

the instance of the mouse and we have used the histopathologic diagnosis solely, since it has the merit of providing a datum which is indeed the decisive one for rendering a verdict of whether or not the disease has been produced.

In the mouse the 100 per cent incidence of EAE was achieved (3) by the following minimal procedure: The young adult mouse of a completely susceptible mouse genotype, the BSVS inbred strain, maintained on a nutritionally complete stock diet, was "primed" with an intraperitoneal injection of *Hemophilus pertussis* vaccine and, 4 days later, was injected intracutaneously with a water-in-oil emulsion of mouse brain proteolipid (Folch and Lees) containing mycobacteria as an adjuvant. This injection was repeated 10 days later and by 30 days after the first proteolipid injection *all* such treated mice exhibited the histopathologic signs of EAE.

The statement that this is the "minimal list" for 100 per cent incidence of mouse EAE infers that to do less than this is to fail. This is reasonable enough and easily confirmed. What is not so evident, but will be shown below, is that disturbance of other more subtle relationships which lie hidden in this "simple" list, can lead to failure too.

That the "minimal list" might contain hidden complexities came to our attention through the publication of Shaw, Fahlberg, Kies, and Alvord (4) of their paper on suppression of EAE in guinea pigs by encephalitogenic proteins extracted from homologous brain. In Fig. 1 of their paper these authors presented without discussion or comment data indicating a novel relationship among the operational entities leading to EAE; *i.e.*, production of EAE was affected adversely not only by a *reduction* in the amount of *Mycobacterium tuberculosis* as adjuvant, but also by its *increase* beyond a certain critical level. In a word, all else being equal, maximal disease as estimated by their disease index was achieved only within a certain range of concentration of mycobacteria in the injected mixtures, and less disease appeared not only with less mycobacteria, but with more.

Since the precise role of the mycobacteria in adjuvant emulsions is not completely understood, it becomes important, on an empirical basis at least, that we define this relationship more precisely. We present here evidence that this paradoxical situation apparently has some general basis, for we have now found a similar set of circumstances in the mouse model; and once we admit this we are led to direct our attention to quantitative aspects of the other items of the emulsion mixture as well.

A multifactorial examination has been made of the consequences for EAE of varying in the injection emulsion (*a*) the mycobacterial species, (*b*) the emulsifier and, for a given mycobacterial species, quantitatively varying (*c*) the mycobacteria and (*d*) the proteolipid antigen.

Methods and Materials

The "Minimal" Procedure.—BSVS mice (5), 2 to 3 months old, on a stock diet of Purina fox chow and whole wheat bread and milk *ad libitum*, were injected intraperitoneally with 0.5 ml *H. pertussis* vaccine (Lederle Laboratories, Pearl River, New York) (phase 1, equivalent to 60,000 million/ml, diluted 1:5 with saline). The following day the backs of the animals were depilated with an electric shaver and on the 4th day the mice were injected intracutaneously in the shaved sites with 0.3 ml mouse brain proteolipid containing mycobacteria

as an adjuvant. 1 ml of the control injection mixture was composed of 14 mg lyophilized mouse brain proteolipid (6) in 0.5 ml 0.85 per cent saline containing 0.2 mg merthiolate homogenized in a Waring blendor with 2.5 mg dead mycobacteria ($H_{37}R_v$) in 0.5 ml soconol (liquid petrolatum, heavy, (USP) distributed by Socony-Vacuum Oil Co., New York). The 0.3 ml dose was divided into 3 blebs through a 1 inch 21-gauge needle. 10 days later a second, similarly divided dose of proteolipid emulsion was injected. The animals were examined daily for signs of EAE. Many neurologic signs were noted, such as flaccid tail, difficulty righting after a spin, weakness of legs, convulsions, circling, but the most reliable sign was paralysis of the legs. On the 20th day after the second proteolipid injection the experiments were terminated. Approximately 24 sections obtained from various parts of each brain were stained with hematoxylin-eosin and the final scoring as positive reactors or non-reactors rested on the histopathologic examination. This procedure has led to EAE in 100 per cent of the cases.

RESULTS

Role of the Mycobacterial Species in the Adjuvant Emulsion.—It had been reported to us from two other laboratories[2, 3] using BSVS mice that there was difficulty in producing EAE with proteolipid. Further examination revealed that the adjuvant used in each of these unsuccessful cases was Difco complete adjuvant (Difco Laboratories, Detroit, Michigan). Difco adjuvant was next tested in our own laboratory. 20 mice in 2 groups were injected as described above with (*a*) proteolipid + our adjuvant, and with (*b*) proteolipid + Difco. In the first group all brains were scored as positive or marked positive. With the Difco adjuvant none of the 10 brains showed any lesions. The Difco adjuvant contains *Mycobacterium butyricum* at a concentration of 0.25 mg per ml. This same concentration of *M. tuberculosis* in the adjuvant emulsion we employ is 100 per cent effective. The failure of the Difco adjuvant in these circumstances might thus be due to a qualititative difference between these two mycobacterial species. This becomes less certain, however, with the recognition that other, and different items of the adjuvant emulsion might be involved, *i.e.*, the emulsifier,[4] the oil base, the size of the emulsion droplets, etc.

In order to explore directly the efficacy of different species of mycobacteria in EAE production, the following experiment was performed.

In samples of 10 BSVS mice each the EAE-producing efficacy of a uniform dose of proteolipid, 14 mg/ml, was determined when this dose was used, without added emulsifier, in conjunction with three different species of mycobacteria: *tuberculosis*,[5] *fortuitum*,[6] and *smegmatis*.[6] Five levels of the first, two levels of

---

[2] Dr. George Clark, Department of Physiology, University of Buffalo, Buffalo.

[3] Dr. Labe C. Scheinberg, Albert Einstein College of Medicine, New York City.

[4] The emulsifier in Difco complete adjuvant is arlacel A (mannide monooleate).

[5] Kindly supplied by Dr. H. D. Piersma, Lederle Laboratories Division, Pearl River, New York.

[6] Kindly supplied by Dr. Curtis A. Williams, Jr., The Rockefeller Institute, New York City.

the second, and two levels of the third species were tested as indicated in Table I. Even with no acid-fast bacteria in the injection emulsion 2 of 10 mice were positive, which agrees with the earlier findings of Bell and Paterson (7). At the level of 0.025 mg mycobacteria/ml the production of EAE was increased and *tuberculosis*, *fortuitum*, and *smegmatis* organisms were almost equally effective. With a 10-fold increase in concentration of mycobacteria, 90 to 100 per cent of the mice showed lesions, and again there was no great difference in the efficacy of the three mycobacteria. The $H_{37}R_v$ strain was again 100 per cent efficient as an adjuvant at the next 10-fold increase. It is important to note that a further increase in concentration resulted in a sharp drop in the production of EAE. The pattern of results produced by *M. tuberculosis* as

TABLE I

Effect on the Production of EAE of Varying the Mycobacterial Species Quantitatively and Qualitatively in the Antigen-Adjuvant Mixture*

Mycobacteria	*M. tuberculosis*	*M. fortuitum*	*M. smegmatis*
mg/ml			
0	2/10‡		
0.025	4/9	3/10	5/10
0.25	10/10	8/9	10/10
2.5	9/9		
25.0	2/9		

* Proteolipid antigen at 14 mg/ml.

‡ Number positive EAE/number injected.

adjuvant exactly parallels that of the Alvord and Kies group (4) alluded to earlier.

Quantitative Interactions between Antigen and Adjuvant Mycobacteria.—The demonstration that for a fixed concentration of proteolipid antigen, 14 mg/ml, the production of EAE was facilitated by an increasing concentration of *M. tuberculosis*, only to be ultimately repressed by an increase beyond an "optimal" concentration, suggested a factorial analysis of the consequences of simultaneously varying the concentrations of antigen and adjuvant mycobacteria (*M. tuberculosis*).

Before the experiment is described a brief comment is necessary on a technical necessity, the addition of an emulsifier to the adjuvant mixture. This addition was necessary because upon reduction of the proteolipid concentration from its usual level of 14 mg/ml the adjuvant emulsion lost the stability provided by the emulsifying properties of this material and the mycobacteria rapidly sedimented on standing. Consequently a commercially available emulsifier, falba,[7] was used for the experiment described below at a concentration of 5 per cent, replacing part of the oil fraction, soconol.

[7] Falba is an absorption base said to be a mixture of "oxycholesterine and cholesterines derived from lanolin" (Pfaltz and Bauer, Inc., New York City).

300 young adult BSVS mice, 150 of each sex, were divided into 30 groups of 10 each, 5 males and 5 females. These groups were used in a 5 × 6 factorial design with adjuvant mycobacteria ranging in concentration from 0 to 25 mg/ml in five 10-fold increments, and with proteolipid ranging from 0.875 mg/ml to 28 mg/ml in 2-fold increments. The 30 mixture combinations thus prepared were injected into the mice in the usual manner and the animals ultimately scored for EAE similarly.

The checker-board style of this experimental design and the results are shown in Table II. It would be redundant to provide a statistical analysis here, for the conclusions are easily reached.

Clearly the quantitative relationships are indeed critical for the production of EAE. With 5 per cent falba as an emulsifier only 1 of 30 combinations of proteolipid antigen and mycobacteria proved to be 100 per cent successful. (14 mg proteolipid and 2.5 mg $H_{37}R_v$ strain/ml). In addition to confirmation

TABLE II

Effect of 5 Per Cent Falba on the Production of EAE

M. tuberculosis	Proteolipid, *mg/ml*					
	0.875	1.75	3.5	7.0	14.0	28.0
mg/ml						
0	0/10*	0/10	0/10	0/10	0/10	0/10
0.025	0/10	0/10	0/10	0/10	0/10	0/10
0.25	0/10	0/10	0/10	0/10	0/10	0/10
2.5	0/10	0/10	6/10	6/10	10/10	5/9
25.0	1/10	0/10	1/10	0/10	1/10	4/10

* Number positive EAE/number injected.

of the previously observed restriction of EAE production by too much (25 mg/ml) or too little (0.25 mg/ml and less) mycobacteria, an analogous situation was found in the case of the antigen. Thus, with mycobacteria held at the 2.5 mg/ml level, a proteolipid concentration of 14 mg/ml provided EAE at 100 per cent effectiveness. But halving the proteolipid antigen, or doubling it, markedly reduced the frequency of EAE.

Effect of the Emulsifier.—The results presented in Tables I and II provided a comparison which now brought into view a previously unanticipated role of the emulsifier in EAE production. Thus, over a range of concentrations of *M. tuberculosis*, with proteolipid fixed at 14 mg/ml, but with emulsifier absent, a certain broad range of EAE production had been observed, (Table I, column 1). Now, in Table II (considering the pertinent column, labeled 14 mg proteolipid/ml) results were presented following the test of these same concentrations of mycobacteria and antigen, but in the presence of an emulsifier (falba). These two experiences are conveniently compared in Table III.

It is evident that the effect of this emulsifier (falba) is to restrict quite

markedly the range of mycobacteria concentrations which lead to EAE production.

That this conclusion had predictive value for other quantitative combinations of antigen and mycobacteria became evident when 110 BSVS young adult mice in 11 sets of 10 each, as indicated in Table IV, were used to test some, though not all, of the combinations tested in Table II. In the present test emulsifier was omitted. This omission meant, of course, a limitation on the lowest level of proteolipid antigen which would provide an adequate emulsification.

The results are presented in Table IV, which furnishes in the shaded blocks the pertinent values obtained with falba for purposes of comparison. The

TABLE III

*Effect on EAE Production of the Presence and Absence of an Emulsifier (5 Per Cent) in the Adjuvant Emulsion**

M. tuberculosis	No emulsifier	With emulsifier (5 per cent falba)
mg/ml		
0	2/10‡	0/10
0.025	4/9	0/10
0.25	10/10	0/10
2.5	9/9	10/10
25.0	2/9	1/10

* Proteolipid antigen included at 14 mg/ml.

‡ Number positive EAE/number injected.

restrictive effects of falba on EAE production are clearly evident over a considerable range of antigen-mycobacteria combinations.

Table IV also presents a further noteworthy relationship between antigen and mycobacteria in the absence of added emulsifier. Starting with 100 per cent EAE in the combination 14 mg/ml proteolipid and 2.5 mg/ml *M. tuberculosis*, a diminution of the antigen (1/8) to 1.75 mg/ml reduced EAE to 50 per cent. But, with the antigen at this new level, reduction of the mycobacteria to 1/10 (0.25 mg/ml) enhanced EAE to the 100 per cent level. Contrariwise, increasing the mycobacteria 10-fold (25 mg/ml) depressed EAE production to 0. This suggests that the critical relationship between antigen and mycobacteria for 100 per cent EAE rests on a balance or ratio basis rather than solely on absolute amounts. This notion receives some further support when another inspection is made using the data of Table IV and Table III. Thus, starting again in the absence of emulsifier at the 14 mg/ml proteolipid, 2.5 mg/ml mycobacteria level, increasing the mycobacteria 10-fold to 25 mg/ml reduced EAE from the 100 per cent level to 22 per cent (Table III), but at this new

level of mycobacteria, a subsequent increase of the antigen to 28 mg/ml restored the EAE to 100 per cent (Table IV).

Qualitative Differences Among Emulsifiers.—The experiments reported above demonstrated the restrictive effects of an emulsifier on the production of EAE in BSVS mice in terms of certain critical quantitative relationships between the antigen and mycobacterial components of the adjuvant emulsion. Thus far, a single such emulsifier had been examined. That there exist certain differences in this regard among the class of emulsifiers which might be used was evident when another emulsifier was tested. Aquaphor[8] was tested at the 5 per cent level in 20 BSVS mice in 2 antigen-mycobacteria combinations in

TABLE IV

Production of EAE in Mice with Proteolipid Emulsions Containing No Added Emulsifier Compared with Emulsions Containing Falba

M. tuberculosis mg/ml		Proteolipid, mg/ml				
		1.75	3.5	7.0	14.0	28.0
0.025	Falba	0/10				0/10
	No Falba	4/9*				4/10
0.25	Falba	0/10				0/10
	No Falba	10/10				10/10
2.5	Falba	0/10	6/10	6/10	10/10	5/9
	No Falba	5/10	10/10	9/10	10/10	14/14
25.0	Falba	0/10				4/10
	No Falba	0/10				10/10

* Number positive EAE/number injected.

which falba had shown to be restrictive for EAE production; *i.e.*, 7 mg and 28 mg/ml of proteolipid and 2.5 mg/ml mycobacteria, respectively. In both of these tests EAE was produced 100 per cent and none of the restrictive effects exhibited under similar circumstances by falba were evident. It would appear unwise, therefore, to indict all emulsifiers in general for their restrictive properties on production of allergic encephalomyelitis. Each, apparently, must be considered on its own merits.

Restriction of EAE Production Considered Operationally.—The preceding experiments provide basis enough to show that the operational specifications necessary for the 100 per cent production of EAE in a completely susceptible genotype must include: (*a*) the presence or absence of emulsifier; (*b*) its quali-

[8] Aquaphor is an ointment base composed of derivatives of wool fat mixed with hydrocarbons (Duke Laboratories, Inc., Stamford, Connecticut).

tative nature, and (*c*) the quantitative relations between proteolipid antigen and adjuvant mycobacteria. The consequences of this for studies in EAE are obvious enough, and need not be labored here. As part of the operational definition of the production of EAE these findings stand on their own. The question is not long in arising, however, just how it is that these relationships discussed above can become so critical as to thwart the attempt to produce the disease. In a word, what goes wrong?

One simple consideration of these matters springs from a kind of mechanical outlook. For example, it will be recalled that the EAE-inducing emulsion is

TABLE V

History of the Injected Intradermal Blebs (0.1 Ml, 3/mouse) of Emulsions of Varied Composition (with 5 Per Cent Falba)

BSVS mice, No.	Emulsion concentrations		EAE		Intact Blebs					
					1st injection sites				2nd injection sites	
	M. tuberculosis	Proteolipid	Prediced, day 21	Found, day 17	Day 2	Day 7	Day 10	Day 17	Day 2	Day 7
	mg/ml	*mg/ml*								
10	0.25	28	0/10*	3/4	30/30‡	30/30	30/30	11/12	18/18	7/12
10	2.5	7	6/10	3/4	30/30	29/30	25/30	12/12	18/18	3/12
10	2.5	14	10/10	4/4	30/30	26/30	20/30	6/12	17/18	5/12
10	2.5	28	5/10	3/3	30/30	30/30	29/30	12/12	18/18	4/12
10	25	7	0/10	0/4	30/30	25/30	10/30	5/12	18/18	2/12
10	25	14	1/10	3/4	30/30	24/30	14/30	0/12	18/18	1/12
10	25	28	4/10	2/4	30/30	19/30	14/30	4/12	18/18	4/12

* Number positive EAE/number injected.

‡ Intact blebs/total number blebs.

injected intradermally to produce depot blebs which must have some role to fulfill in the pathogenesis of the disease. If an added emulsifier, or a change in the ratio of antigen and adjuvant mycobacteria, results in a reduction of the frequency of EAE, it is conceivable that the duration and integrity of these depot blebs were thereby altered and we need look no further for an explanation.

To examine this possibility 70 BSVS young adult mice were injected, in groups of 10, with seven emulsions, all containing 5 per cent falba emulsifier, but with various ratios of antigen proteolipid and adjuvant mycobacteria, as indicated in Table V, with predictable departures from, and including, the 100 per cent effective EAE combination. During the usual course of dual injections the depot blebs of emulsion on the shaved backs of the mice were examined daily and on the 10th, 12th, and 17th day the skin sites were excised for histologic examination from 4, 2, and 4 animals per group, respectively. The experiment was terminated on the 17th day and brains were removed for histologic examination. Results are presented in Table V.

The data presented in Table V do not provide any support for a simple mechanical explanation, based on the integrity of the injected blebs. All bleb depots were intact for 2 days and more. As Freund (8) showed, an intradermally delivered antigen-adjuvant mixture can be excised 1 day after its establishment without affecting the immunologic consequences; Freund and Lipton (9) showed this to be directly applicable in EAE in guinea pigs. In the present instance it can therefore be inferred that the different dose mixtures were comparably delivered and maintained long enough so that disparate consequences were not to be assigned to disparate beginnings. The ultimate ulceration of some of these sites occurred with greater frequency in the instances of the higher concentration of *M. tuberculosis*, but these events occurred late and were not correlated with the restriction of EAE. Apparently, whether a bleb ultimately ulcerates to the exterior is an event influenced somewhat by mycobacterial concentration, but it may also reflect the technical variation in the depth of the intradermal injection.

The hypersensitivity induced by the first injection is readily apparent from the shorter duration to ulceration in the second injection series.

Histologic examination of the excised skin sites failed to reveal any features allowing one to differentiate between events in the skin of a mouse in which EAE was ultimately to appear or in which it was destined not to appear. Figs. 1 to 4 show, for example, skin sites on the 17th day following the first injection of emulsion. The similarity of these two sections is noteworthy since the mouse in Fig. 1 had EAE as diagnosed by brain section and the mouse in Fig. 2 did not. This observed disparity in EAE fulfilled the prediction based on the composition of the antigen-adjuvant emulsion.

DISCUSSION

Freund's adjuvant emulsions, in spite of their empiricism, have had an increasing use both in animals and man over the last 20 years, and for the best of reasons: they succeed in enhancing immunologic responses both in extent and in duration. This is not the place to review the history of Freund's important discovery and the phenomenology which has come to surround it (8). But since we have been led in this paper to examine certain quantitative relations between the antigen and mycobacterial concentrations the question arises whether the issue has arisen on previous occasions. Freund, in a review (10), directed attention to the importance of oil-and-water ratios, especially when falba was used as an emulsifier. This is noteworthy since we have found in the present experiments that falba has restricting properties tending to diminish the chances of immunologic success. But the question of the possible critical quantitative relations between antigen and mycobacteria does not appear to have been systematically attacked before.

If our analysis pointing to the necessity of operational specification of

proteolipid and mycobacterial concentrations in their mutual relations in production of EAE is correct, then certain consequences follow:

(*a*) Failure to produce EAE in susceptible genotype in searches for antigenic fractions of brain substance may be due to the possibility that an unsuccessful ratio of antigen to mycobacteria was employed, rather than that the material was truly non-antigenic.

(*b*) Experiments in which host susceptibility is manipulated must be designed to include antigen-mycobacterial ratios over an empirically determined range. It is conceivable that a host change may reduce the frequency of EAE in one test situation and the same manipulation result in increased frequency of EAE in another. In our laboratory we have observed just such puzzling events on several occasions.

(*c*) Originally observed in the guinea pig model of EAE (4), and now systematically investigated in the mouse EAE model, the crucial role of the antigen-mycobacterial relationship must be considered to be general and not restricted to a single host species. Whether such critical relations are to be found in other antigen-adjuvant systems as well must be left for further investigation to determine.

(*d*) Titration of antigen by dilution in emulsions with fixed mycobacterial concentration will, of course, result in an altered ratio of these components. The reduced efficacy of the antigen, it must now be considered, may well reflect this unbalanced ratio rather than the mere reduction of antigenic mass. Indeed, as our experiments have now shown, the declining efficacy of the antigen upon dilution can be restored to the full by proportionally reducing the mycobacterial concentration and restoring the original ratio. It should be remarked that, *a priori*, there must be some lower and finite limit to this diminution of antigen and mycobacteria in fixed ratio, but in these experiments it has not been systematically sought or found.

Speculation on the basis of the altered EAE frequencies following alteration of the antigen-mycobacterial ratios would appear to follow two paths. Either we are confronted with aspects of antigen competition or these changing ratios result in important changes in the water-oil phase concentrations of the antigen The restrictive role of emulsifier, possibly quantitatively affected by the qualitative nature of the emulsifier, would fall into the latter range of speculative possibilities.

SUMMARY

The production of EAE in the fully susceptible BSVS mouse genotype has been found to be dependent on the ratio of proteolipid antigen and adjuvant mycobacterial concentration as used in the emulsion of the Freund type. Disturbance of this ratio, by manipulation of either component, by diminution or increase, results in a decrease in the frequency by which EAE is produced.

Simultaneous reduction of antigen and mycobacteria, so that the ratio remains unchanged, retains the full EAE-producing power of the emulsion. The limit of this has not been ascertained.

Emulsifying agents have been found to restrict further the permissible limits of the antigen-mycobacterial ratio for full EAE production. Such effects of the emulsifier have been found to vary with the qualitative nature of the emulsifier. Aquaphor has been found to be less restrictive than falba.

These phenomena, systematically analyzed here for the mouse, may have an application for other antigen-adjuvant systems and for other hosts.

BIBLIOGRAPHY

1. Kies, M. W., and Alvord, E. C., Jr., "Allergic" Encephalomyelitis, (M. W. Kies and E. C. Alvord, Jr., editors) Springfield, Illinois, Charles C. Thomas, 1959.
2. Alvord, E. C., Jr., and Kies, M. W., Clinico-pathologic correlations in experimental allergic encephalomyelitis. II. Development of an index for quantitative assay of encephalitogenic activity of "antigens," *J. Neuropath. and Exp. Neurol.*, 1959, **18,** 447.
3. Lee, J. M., and Olitsky, P. K., Simple method for enhancing development of acute disseminated encephalomyelitis in mice, *Proc. Soc. Exp. Biol. and Med.*, 1955, **89,** 263.
4. Shaw, C.-M., Fahlberg, W. J., Kies, M. W., and Alvord, E. C., Jr., Suppression of experimental "allergic" encephalomyelitis in guinea pigs by encephalitogenic proteins extracted from homologous brain, *J. Exp. Med.*, 1960, **111,** 171.
5. Lee, J. M., Olitsky, P. K., Schneider, H. A., and Zinder, N. D., Role of heredity in experimental disseminated encephalomyelitis in mice, *Proc. Soc. Exp. Biol. and Med.*, 1954, **85,** 430.
6. Folch, J., and Lees, M., Proteolipides, a new type of tissue lipoproteins. Their isolation from brain, *J. Biol. Chem.*, 1951, **191,** 807.
7. Bell, J., and Paterson, P. Y., Rapid induction of allergic encephalomyelitis in rats without the use of mycobacteria, *Science*, 1960, **131,** 1448.
8. Freund, J., The effect of paraffin oil and mycobacteria on antibody formation and sensitization. A review., *Am. J. Clin. Path.*, 1951, **21,** 645.
9. Freund, J., and Lipton, M. M., Experimental allergic encephalomyelitis after the excision of the injection site of antigen-adjuvant emulsion., *J. Immunol.*, 1955, **75,** 454.
10. Freund, J., Some aspects of active immunization, *Ann. Rev. Microbiol.*, 1947, **1,** 291.

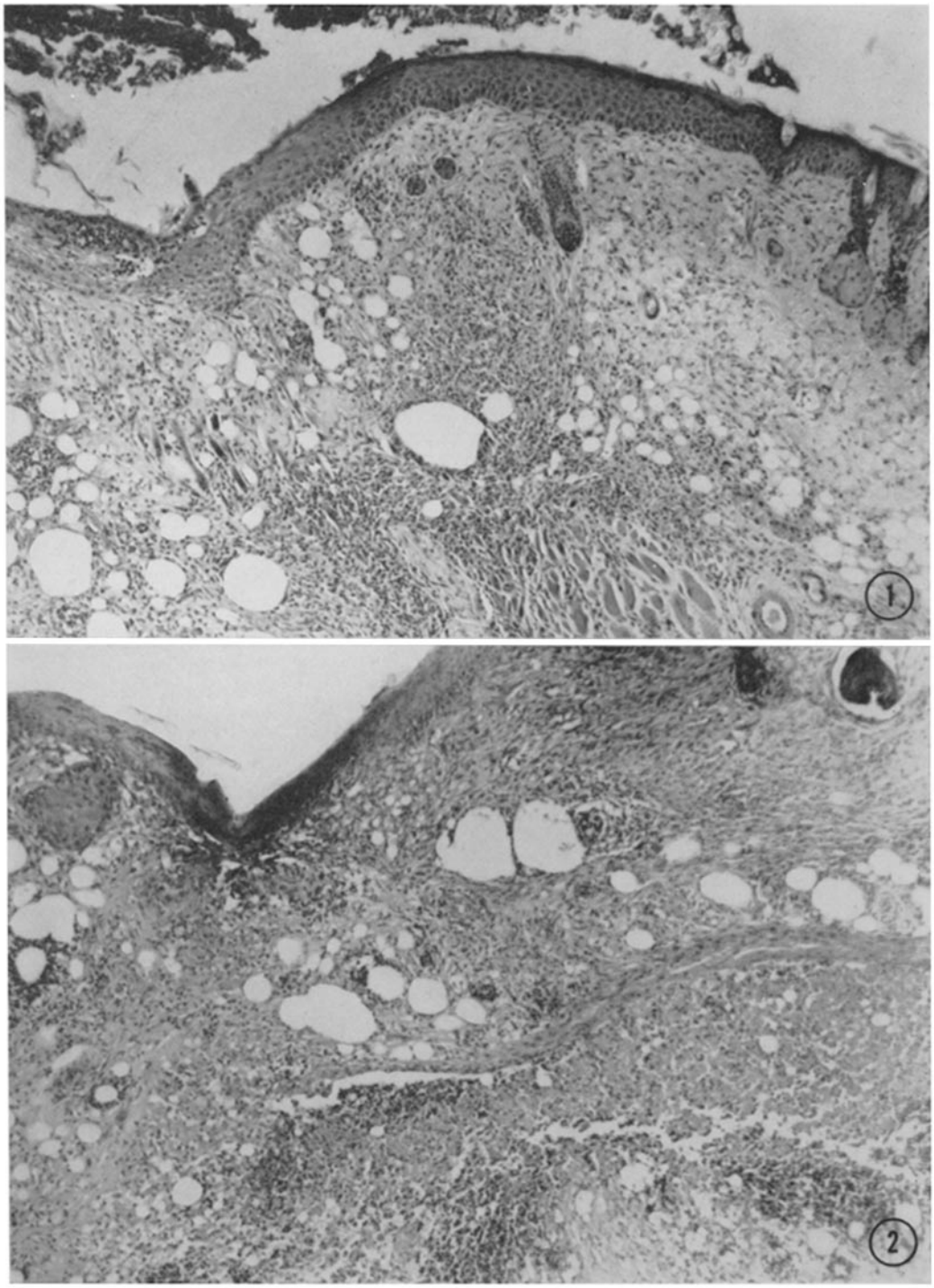
EXPLANATION OF PLATES

PLATE 20

FIGS. 1 to 4. Photomicrographs of granulomatous lesions in the skin of BSVS mice injected intradermally with an emulsion containing proteolipid, *M. tuberculosis*, and 5 per cent falba. These nodules were excised on day 17 after inoculation. To be noted is the insignificant difference in the extent and character of the lesions.

FIG. 1. Nodule of a mouse injected with emulsion containing 14 mg proteolipid/ml and 2.5 mg *M. tuberculosis*/ml. This mouse was scored as EAE-positive. × 103.

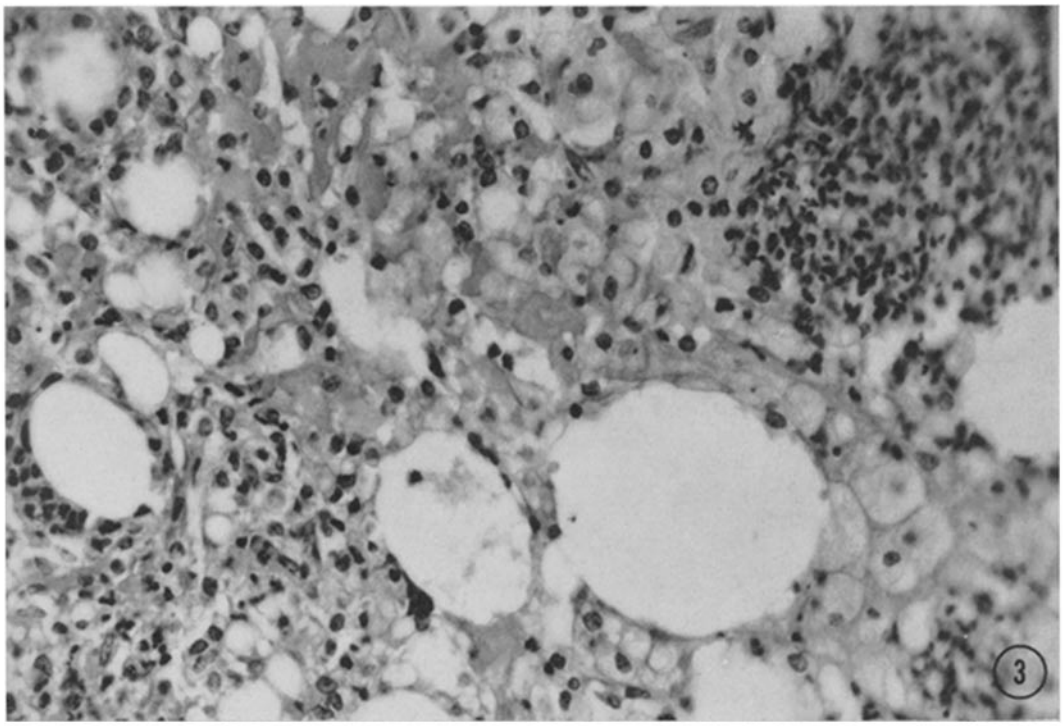
FIG. 2. Emulsion injected into this mouse contained the same amount of proteolipid as in that in Fig. 1 but ten times as much *M. tuberculosis* (25 mg/ml). This mouse was scored as EAE-negative. × 103.

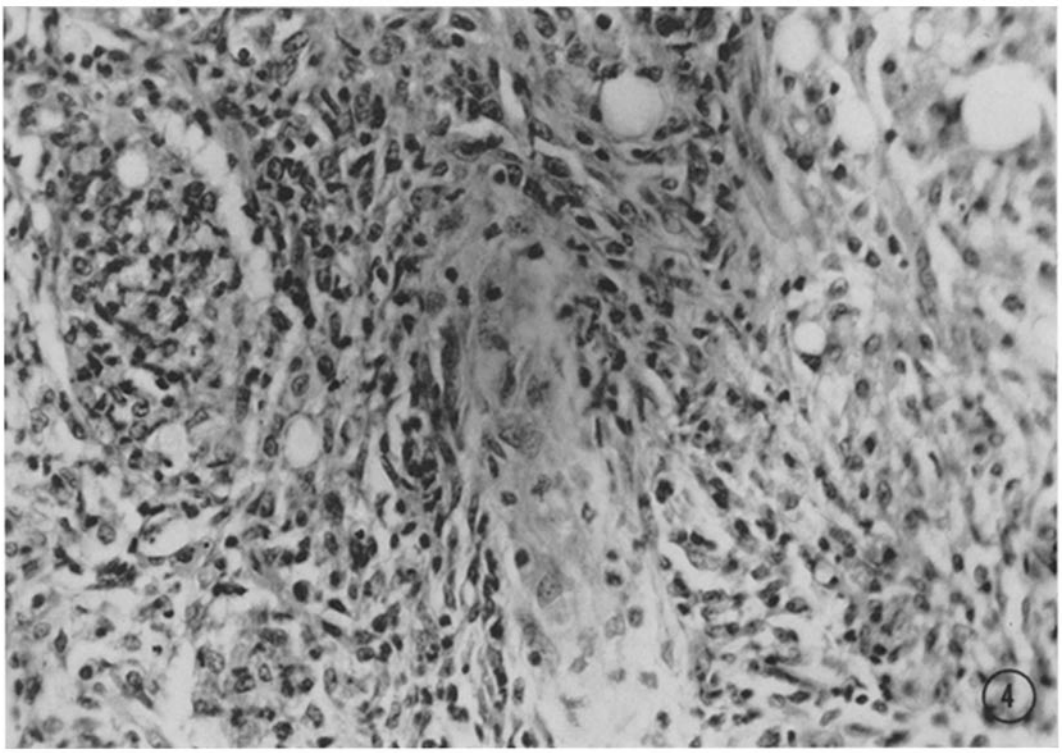


(Lee and Schneider: Antigen-adjuvant composition in EAE)

PLATE 21

FIGS. 3 and 4. These are higher magnifications of Figs. 1 and 2, respectively, showing the similarity in the type of cellular reaction. × 450.





(Lee and Schneider: Antigen-adjuvant composition in EAE)