

# Potentiating Effect of *Nocardia asteroides* on Sensitization to Picryl Chloride and on Production of Isoallergic Encephalomyelitis.

JULES FREUND AND MURRAY M. LIPTON.

From the Division of Applied Immunology, the Public Health Research Institute of the City of New York, Inc.

It has been shown that antibody formation is enhanced when antigens are administered in water-in-oil emulsions and that the effect on antibody formation is not associated with a conspicuous alteration of sensitization. The addition of killed mycobacteria to antigens in water-in-oil emulsions, however, has two effects; it alters sensitization and further potentiates antibody formation.<sup>1</sup> Both of these effects as caused by living *M. tuberculosis* in tuberculous animals had been observed by Lewis and Loomis,<sup>2</sup> and Dienes.<sup>3</sup>

Examples of the potentiating effect of killed mycobacteria in water-in-oil emulsions are numerous. They include with regard to sensitization the delayed type (tuberculin type) of sensitization to equine serum,<sup>4</sup> contact type of sensitization to picryl chloride<sup>5</sup> and the production of isoallergic encephalomyelitis;<sup>6</sup> with regard to antibody formation the production of agglutinins against *S. typhosa*,<sup>7</sup> neutralizing antibodies against influenza virus<sup>8</sup>

and neutralizing and complement fixing antibodies against rabies virus.<sup>9</sup> The relationship of adjuvants to immunization to malaria is particularly interesting. It is possible to immunize rhesus monkeys against infection with *P. knowlesi* by killed parasites in water-in-oil emulsion containing killed *M. tuberculosis* but identical vaccine without mycobacteria is ineffective. Furthermore, a lipid extract from tubercle bacilli which enhances antibody formation against *S. typhosa* is ineffective as a substitute for the whole mycobacterial cell with regard to immunization against malaria.<sup>10</sup> This observation increased our interest in the nature of the synergistic effect of mycobacteria.

It is hoped that light may be shed on the mode of action of mycobacteria and on immune-response in general by finding other agents endowed with similar adjuvant properties. The purpose of the present paper is to report on the effect of killed *Nocardia asteroides* (actinomyces) on sensitization to picryl chloride and the production of isoallergic encephalitis.

**Sensitization to picryl chloride.** Landsteiner and Chase<sup>5</sup> found that the skin of the guinea pig can be sensitized (contact type of sensitization) to this simple compound by injecting a picryl conjugate of guinea pig stromata into the peritoneal cavity, provided that killed tubercle bacilli in paraffin oil were likewise injected into the peritoneal cavity; later, the sensitization was found to be more satisfactorily attained by a single subcu-

<sup>1</sup> Freund, J., and Walter, A. W., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 47; Freund, J., *Ann. Rev. Microbiology*, 1947, **1**, 291.

<sup>2</sup> Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, **40**, 503.

<sup>3</sup> Dienes, L., *J. Immunol.*, 1928, **15**, 153.

<sup>4</sup> Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.

<sup>5</sup> Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1940, **71**, 237.

<sup>6</sup> Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131; Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117; Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179; Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229; Morrison, L. A., *Arch. Neur. and Psych.*, 1947, **58**, 391.

<sup>7</sup> Freund, J., and Bonanto, M. V., *J. Immunol.*, 1944, **48**, 325.

<sup>8</sup> Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.

<sup>9</sup> Freund, J., and Lipton, M. M., unpublished experiments.

<sup>10</sup> Freund, J., Thomson, K. J., Sommer, H. E., Walter, A. W., and Pisani, T. M., *Am. J. Trop. Med.*, 1948, **28**, 1; Freund, J., Thomson, K. J., Hough, H. B., and Pisani, T. M., *J. Immunol.*, in press.

TABLE I.  
Sensitization to Picryl Chloride.

| Material injected    | Group A | Group B | Group C |
|----------------------|---------|---------|---------|
| Picryl stromata      | 10 mg   | 10 mg   | 10 mg   |
| Saline solution      | 0.6 ml  | 0.6 ml  | 0.6 ml  |
| Falba                | 0.5 "   | 0.5 "   | 0.5 "   |
| Paraffin oil         | 0.6 "   | 0.6 "   | 0.6 "   |
| <i>N. asteroides</i> | 5 mg    | 1 mg    | 0 mg    |
| Guinea pig No.       |         |         |         |
| 1                    | 5+      | 5+      | ±       |
| 2                    | 5+      | 5+      | tr.     |
| 3                    | 5+      | 5+      | 0       |
| 4                    | 4+      | 5+      | 0       |
| 5                    | 4+      | ±       | 0       |
| 6                    | 2+      | trace   | 0       |
| 7                    | ±       |         | 0       |
| 8                    | ±       |         | 0       |
| 9                    |         |         | 0       |
| 10                   |         |         | 0       |

5+ = bright red, markedly elevated.

4+ = pink or bright pink, elevated.

2+ = pale pink, slightly elevated.

± = faint pink, small spots.

trace = very faint pink, small spots.

0 = no discoloration, no elevation.

taneous injection of picryl guinea pig stromata incorporated into a water-in-oil emulsion containing killed tubercle bacilli.<sup>11</sup> No sensitization (or occasionally a feeble one) occurs in the absence of mycobacteria.

To test the effect of *N. asteroides*, guinea pigs were injected with picryl guinea pig stromata\* emulsified in paraffin oil containing killed fungi instead of mycobacteria. The strain of *N. asteroides* (American Type Culture Collection No. 9969) is not acid-fast by the Ziehl-Neelsen technic but retains carbol fuchsin when treated with 1% aqueous H<sub>2</sub>SO<sub>4</sub> and washed with water. The fungi were collected from Sabouraud's agar, autoclaved and dried over P<sub>2</sub>O<sub>5</sub>. The animals received 5 simultaneous deep subcutaneous injections, a total of 1.7 ml of the emulsions; the material injected is given in Table I. The technic of the preparation of emulsion was given in previous papers.<sup>6</sup> Sensitization was tested 31 days after the injection by placing 1 drop of a 1% picryl chloride solution in olive oil on the skin. The reactions were read 1 and 2 days later. Table I shows that killed *N. asteroides* was effective as a potentiating agent.

<sup>11</sup> Chase, M. W., personal communication.

\* Obtained through the courtesy of Dr. Merrill W. Chase.

*Production of isoallergic meningo-encephalitis.* Rivers and associates<sup>12</sup> succeeded in producing allergic encephalitis by frequently repeated injections of brain extracts or suspensions of autolysed brain without adjuvants. A single injection of normal brain is, however, sufficient if the brain is incorporated in a water-in-oil emulsion containing killed *M. tuberculosis* or *M. butyricum*.<sup>6</sup> In the absence of mycobacteria the rapid appearance of the phenomenon fails to occur and certain lipid extracts from tubercle bacilli are ineffective in this respect.<sup>13</sup>

To test the effect of *N. asteroides* as a substitute for mycobacteria, 3 groups of guinea pigs were given 3 simultaneous deep subcutaneous injections of fresh guinea pig brain in water-in-oil emulsion containing killed fungi.

The material injected and the results are given in Table II. *N. asteroides* was at least as effective as *M. tuberculosis* or *M. butyricum*. The neurological symptoms and histological lesions of the central nervous system were similar to those found in guinea pigs injected with brain, paraffin oil and killed mycobacteria.<sup>6</sup> Groups of 18 mice received 0.2 ml of an emulsion containing 0.08 ml of a 10% suspension of mouse brain in saline, 0.04 ml Falba, 0.08 ml paraffin oil and 0.2 mg (dry weight) killed *N. asteroides*, or the same material but rabbit brain instead of mouse brain. No neurological symptoms of encephalitis were observed during 1 month as in similar experiments with mycobacteria.<sup>6</sup>

*Sensitization to N. asteroides and to tuberculin.* It can be expected that the killed fungi suspended in paraffin oil will produce specific fungi was extracted with 30 ml of 1/20 skin sensitization of the delayed type. The sensitization was tested with an alkaline extract of the partially defatted fungi. Fluid medium cultures were treated with 0.1%

<sup>12</sup> Rivers, T. M., Sprunt, D. M., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39; Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559; Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.

<sup>13</sup> Freund, J., and Lipton, M. M., unpublished experiments.

TABLE II.  
Isoallergic Meningo-encephalitis in Guinea Pigs.

| Material injected<br>per guinea pig | Guinea pig<br>No.* | Paralysis<br>day of onset | Paresis<br>day of onset | Dead or killed<br><i>in extremis</i> ,<br>days | Histological<br>lesions<br>in CNS |
|-------------------------------------|--------------------|---------------------------|-------------------------|------------------------------------------------|-----------------------------------|
| Guinea pig brain 0.1 g              | 1                  | 23                        |                         | K25                                            | Present                           |
| Saline sol. 0.9 ml                  | 2                  | 22                        |                         | K25                                            | "                                 |
| Falba 0.5 "                         | 3                  | 24                        |                         | K25                                            | "                                 |
| Paraffin oil 1 "                    | 4                  | 24                        |                         | K25                                            | "                                 |
| <i>N. asteroides</i> 10 mg          | 5                  |                           | 24†                     | Living‡                                        |                                   |
|                                     | 6                  |                           | 24†                     | " ‡                                            |                                   |
|                                     | 7                  | 36                        |                         | K43                                            | Absent                            |
|                                     | 8                  |                           | 56 and 122†             | Living‡                                        |                                   |
|                                     | 9                  |                           | 25†                     | " ‡                                            |                                   |
|                                     | 10                 |                           |                         | " ‡                                            |                                   |
|                                     | 11                 |                           |                         | D62                                            | "                                 |
|                                     | 12                 | 20                        |                         | K24                                            | Present                           |
|                                     | 13                 |                           |                         | D38                                            | Absent                            |
|                                     | 14                 | 13                        |                         | K19                                            | Present                           |
| Guinea pig brain 0.1 g              | 15                 |                           |                         | D22                                            | Absent                            |
| Saline sol. 0.9 ml                  | 16                 |                           | 24                      | D27                                            | Present                           |
| Falba 0.5 ml                        | 17                 | 19                        |                         | K20                                            | "                                 |
| Paraffin oil 1 "                    | 18                 |                           | 16                      | K16                                            | "                                 |
| <i>N. asteroides</i> 1.0 mg         | 19                 | 23                        |                         | K24                                            | "                                 |
|                                     | 20                 | 19                        |                         | K24                                            | "                                 |
|                                     | 21                 |                           | 15                      | D16                                            | "                                 |
|                                     | 22                 | 20                        |                         | K22                                            | "                                 |
|                                     | 23                 | 28                        |                         | K31                                            | "                                 |

\* Group I, No. 1 through 10; Group II, No. 11 through 14; Group III, No. 15 through 23.

† Transient.

‡ 133 days.

formalin, the fungi extracted with acetone, later with equal parts of 95% ethanol and ether and dried over  $H_2SO_4$ . One gm of these fungi was extracted with 30 ml of 1/20 normal NaOH for 2 hours and centrifugalized. The supernate was neutralized with 1 normal HCl.

The guinea pigs in the picryl chloride experiment were skin-tested 6 weeks after the original injection. All except the controls (Group C) reacted to a 1:2 dilution of the alkaline extract with redness, elevation and necrosis. The reactions varied from about 20 to 30 mm in diameter and lasted for more than 2 days. It may be noted that the aqueous extract produced a slight redness in the skin of the controls but this reaction diminished during the first day. Eleven weeks after the original injection 5 animals of the encephalitis experiment were skin-tested with 0.1 ml saline containing 0.1 mg killed *N. asteroides*. The reactions were severe and were of the delayed type.

Besides the successful use of *N. asteroides* reported in the present paper allergic encephalitis has been produced with the aid of myco-

bacteria, *M. tuberculosis* and *M. butyricum*. Both are equally effective and both sensitize to tuberculin although the latter to a lesser degree than the former.

It was desirable to learn whether guinea pigs injected with *N. asteroides*, an actinomycete biologically close to mycobacteria, would likewise be made sensitive to tuberculin. Drake and Henrici<sup>14</sup> reported that living *Nocardia* suspended in paraffin oil do not induce sensitization to tuberculin, although previously Goyal<sup>15</sup> had found that strains of *Nocardia* sensitize guinea pigs to tuberculin. Goyal does not mention the degree of sensitization. For this investigation, skin-tests were made with an Old Tuberculin prepared from a culture grown on a synthetic medium<sup>†</sup>

<sup>14</sup> Drake, C. H., and Henrici, A. T., *Am. Rev. Tub.*, 1943, **48**, 184.

<sup>15</sup> Goyal, R. K., *Ann. Inst. Pasteur*, 1937, **59**, 94.

† This tuberculin, prepared from growths of human strains of tubercle bacilli on Dorset's Synthetic Medium, was sent to Dr. M. W. Chase from the Reichel Division of Wyeth, Inc., through the courtesy of Dr. B. Scott Fritz.

and with an Old Tuberculin prepared from a glycerin veal infusion broth; the test doses ranged from 0.1 ml of a 1:20 dilution to 0.1 ml of a 1:100 dilution. Actually, it was found that killed *Nocardia* do sensitize to tuberculin, but the sensitization is usually feeble; it is necessary to use rather high concentrations of tuberculin and to make the test some time (5 or 6 weeks) after the primary sensitizing injection.

Details of the reactions follow: All of the picryl chloride animals which received fungi reacted to a 1:20 dilution of old tuberculin, prepared on a synthetic medium. Seven weeks after the injection of the emulsion the following reactions were observed: After 24 hours in Groups A and B pinkishness and elevation ranging from 10 mm to 20 mm in diameter with blanching; at 48 hours the reactions ranged from negative to ill-defined pinkishness and elevation 18 mm in diameter; controls (Group C) had very slight reactions at 24 hours which became negative at 48 hours. The same OT was diluted 1:80 and 4 guinea pigs of groups A and B were tested. The reactions at 24 hours ranged from very ill-defined pinkishness 10 mm to well-defined redness and induration 14 mm in diameter. At 48 hours the reactions were doubtful to ill-defined faint pinkishness and elevation 12 mm in diameter. Eight guinea pigs of groups A and B were tested with a 1:20 dilution of OT prepared on broth. The reactions were stronger and some of the animals had necrotic reactions.

The animals of the encephalitis experiment were tuberculin-tested at different intervals of time following the injection of brain and fungi and oil. After 21 days Groups II and III and after 44 days 5 survivors in Group I failed to react to a 1:20 dilution of OT. The latter 5 pigs were re-tested 93 days after the injection of the emulsion. This time they reacted to a 1:20 dilution of OT prepared on synthetic medium. The reactions ranged from ill-defined pinkishness and elevation  $10 \times 6$  mm to well-defined pinkishness and elevation 18 mm in diameter with slight blanching at 24 hours, and at 48 hours were negative to well-defined pink and elevation 16 mm in

diameter. Two days later the injection of OT (prepared on broth) in 1:20 dilution resulted in similar reactions. With a dilution of 1:100 at 24 hours the reactions to OT ranged from faint pinkishness and elevation 10 mm to well-defined pinkishness and elevation 13 mm in diameter. These reactions were much weaker at 48 hours. The reactions to 1:1000 OT were doubtful after 24 and negative after 48 hours.†

Killed *N. asteroides* in water-in-oil emulsion produces a cellular reaction in the subcutaneous tissue which is similar to that found with mycobacteria.<sup>6</sup> It seems, however, that giant cells are less numerous and more of the foreign body type, and the granulation tissue with *N. asteroides* is richer in blood vessels. Like killed mycobacteria, killed *N. asteroides* in water-in-oil emulsion produces a delayed type of specific sensitization. In addition *N. asteroides* sensitizes to tuberculin. However, killed *M. tuberculosis* in paraffin oil sensitizes to very high dilutions of tuberculin up to 1 in 10,000 whereas *N. asteroides* in paraffin oil produces only a slight sensitization to tuberculin. The potentiating effect of *M. tuberculosis* and *N. asteroides* may depend on the cellular reaction these organisms produce. The slight degree of sensitization to tuberculin by *N. asteroides* might be incidental.

Experiments on the effect of *N. asteroides* on the production of antibodies against *S. typhosa* indicate that this organism, unlike mycobacteria, does not enhance agglutinin formation when combined with typhoid vaccine in oil emulsion.

**Summary.** 1) Killed *N. asteroides* can replace mycobacteria as an adjuvant in the production of (1) skin sensitization to picryl chloride, and (2) isoallergic meningo-encephalitis. 2) Killed *N. asteroides* in water-in-oil emulsion induces a delayed type of sensitization to an extract from this fungus. 3) Killed *N. asteroides* in water-in-oil emulsion sensitizes to tuberculin. The degree of sensitization is slight when compared with that produced by killed *M. tuberculosis* in oil.

**SUBSEQUENT NOTE:** Demyelination, conspicuous about blood vessels in the CNS, was

† Skin tests with commercial streptomycin preparations were negative.

demonstrated by Spielmeyer's stain. It was found in paralyzed guinea pigs using *N. asteroides* or *M. tuberculosis* or *M. butyricum* as potentiating agents.

Advice received from Dr. L. Raymond Morrison, Department of Neuropathology, Massachusetts General Hospital, is gratefully acknowledged.

## 16491 P

### Effect of Urethane, X-rays, Potassium Arsenite, Benzol, on Survival Time in Transplanted Mouse Leukemia.\*

ARTHUR KIRSCHBAUM, SISTER TERESITA JUDD, CHEN SHAN LU,<sup>†</sup> RUBY M. ENGSTROM, AND HARRY MIXER.

From the Departments of Anatomy and Radiology, University of Minnesota Medical School, Minneapolis.

Urethane depresses the white blood cell count and decreases the size of infiltrated organs in certain cases of human leukemia.<sup>1</sup> Clinical improvement was more striking in chronic myeloid than in chronic lymphatic and acute leukemia.<sup>2</sup> Certain transplanted and spontaneous rodent leukemias were susceptible to the effects of urethane.<sup>3-6</sup>

In these experiments 5 transfer lines of mouse leukemia were studied. Four originated in the high leukemia F strain<sup>7</sup> and a fifth (line RE) was transplanted originally from a spontaneous case which arose in an F<sub>1</sub> hybrid mouse between strains F and NH. Three of these lines of leukemia (15, 686, and RE) were myeloid, and 2 (86 and 291) were

lymphoid. The routine method of transfer was by intraperitoneal inoculation of 5,000-10,000 splenic cells suspended in isotonic saline. Inoculated mice were all F<sub>1</sub> hybrids (strain F × strain NH) approximately 6 weeks of age.

The methods of treatment with urethane were the following:

a) At the time of transfer an anesthetic dose was administered subcutaneously with no further treatment, or

b) 2, 3 or 4 anesthetic doses were given daily for the first 2, 3 or 4 days. No further treatment was given to these mice, or

c) one-third or one-fifth of an anesthetic dose (1 mg/g) was given daily from the time of inoculation, or

d) after inoculated mice had become leukemic, 2 daily anesthetic doses were given, these being followed by daily doses of 0.33 mg/g body weight.

Survival was significantly prolonged in transplanted myeloid leukemia by all methods of treatment with urethane except the single anesthetic dose at the time of transfer, and 2 doses in the first 2 days following transfer (see Table I); in 2 instances 2 doses increased survival.

Line 291 was a relatively chronic lymphatic leukemia. Although the white blood cell count was depressed by urethane, survival was not appreciably prolonged by the same type of treatment effective for the lines of myeloid leukemia. Lymphoid line 86 responded similarly to line 291 (see Table I for data).

\* This investigation has been aided by grants from the Donner Foundation, the Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute, and the Graduate Medical Research Fund of the University of Minnesota.

<sup>†</sup> Fellow of the China Medical Board.

<sup>1</sup> Paterson, E., Haddow, A., ApThomas, I., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

<sup>2</sup> Hirschboeck, J. S., Lindert, M. C. F., Chase, J., and Calvy, T. L., *J. A. M. A.*, 1948, **136**, 90.

<sup>3</sup> Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

<sup>4</sup> Weir, D. R., and Heinle, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 268.

<sup>5</sup> Law, L. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 158.

<sup>6</sup> Murphy, J. B., and Sturm, E., *Science*, 1946, **104**, 427.

<sup>7</sup> Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.