Lippincott, Philadelphia (1965).

11. Leblond, C. P., Wilkinson, G. W., Belanger, L. F., and Robichon, J., Am. J. Anat. 86, 283 (1950).

12. Hac, L. R. and Freeman, S. Am. J. Physiol. 212, 213 (1967).

13. Wharton, H. W., Anal. Chem. 34, 1296 (1962); 35, 406 (1963).

14. Suttie, J. W. and Phillips, P. H., Arch. Biochem Biophys. 83, 355 (1959).

15. Hodge, H. C., Metab. Interrelations, Trans. Conf. 4, 250 (1952).

16. Zipkin, I. and Scow, R. O., Am. J. Physiol. 185, 81 (1956).

17. Wallace-Durbin, P., J. Dental Res. 33, 789 (1954).

18. Posner, A. S., Eanes, E. D., and Zipkin, I., "Calcified Tissues" (L. J. Richelle and M. J. Dallemagne, eds.), p. 79. Collection des colloq. Univ. de Liege (1965).

Received July 22, 1968. P.S.E.B.M., 1969, Vol. 130.

# The Adjuvant Effect of Pertussis Vaccine in Experimental Thyroiditis of the Rat* (33573)

FRANK J. TWAROG AND NOEL R. ROSE

*Department of Microbiology, State University of New York at Buffalo School of Medicine, Buffalo, New York 14214*

*Bordetella pertussis* vaccine was first shown to increase the encephalitogenic potency of a central nervous system antigen–complete Freund adjuvant emulsion in mice by Lee and Olitsky (1) and later in rats by Levine and Wenk (2). Subsequent work revealed that the potentiating action of pertussis vaccine was such that it could effectively replace complete Freund adjuvant in the sensitizing injection (3, 4). Because of the impressive results obtained using pertussis vaccine in the allergic encephalomyelitis system, we investigated its effect on the production of another autoimmune disease in rats, experimental thyroiditis. Parallel findings have been obtained by Paterson and Drobish (5) who observed that allogeneic thyroid extracts plus complete Freund adjuvant together with pertussis induced thyroiditis within 3 weeks in 100% of either Lewis or CFN rats. Allogeneic or xenogeneic extracts without pertussis gave little or no thyroiditis at 16 or 21 days in either test strain.

*Material and Methods.* Female Lewis (Microbiological Associates, Inc., Bethesda, Md.) and Fischer 344 (Simonsen Labs, Gilroy, Calif.) rats between the ages of 6–12 weeks were used in the following experiments. Saline extracts were prepared from random bred Wistar rat thyroids (Pel Freez Biologicals, Rogers, Arkansas). The thyroids were shipped in the frozen state and were stored in a freezer until the time when extract was prepared. These antigen preparations (RaTE) were adjusted to a protein concentration of 30 mg/ml.

The immunization regimen consisted of a single injection of 0.2 ml of RaTE (6 mg) emulsified in an equal volume of complete Freund adjuvant. The complete Freund adjuvant was prepared from incomplete Freund adjuvant (Difco) with the addition of 4 mg/ml of *Mycobacterium tuberculosis* $H_{37}Ra$. This material was administered intradermally in the four foot pads and intramuscularly in the thighs. Where noted a simultaneous injection of 0.5 ml of *Bordetella pertussis* vaccine was given on the dorsum of each foot. The pertussis vaccine was an aqueous preparation containing 200 billion organisms/ml.

Serum antibody titers were measured in a microtitration system using the tanned cell hemagglutination procedure of Rose and Witebsky (6). Upon sacrifice of the animals, the portion of trachea containing thyroid tissue was removed *in toto* and fixed in a phosphate buffered formalin solution. Histological sections were prepared and stained with he-

* Supported in part by US Public Health Service Grant CA-05203, National Cancer Institute, National Institutes of Health.

TABLE I. Immunization of Lewis Rats with Rat Thyroid Extract in Complete Freund Adjuvant.

| Duration (days) | Hemagglutination titer | | No. of animals with thyroid pathology[a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Range | — | ± | + | 2+ | 3+ | 4+ |
| 5 | 0 | 0 | 6 | | | | | |
| 10 | 48 | 4–256 | 5 | | | | | |
| 11 | 2 | 0–8 | 2 | | 4 | | | |
| 14 | 8 | 0–64 | 15 | 4 | 1 | 1 | 1 | |
| 17 | 6 | 0–64 | 2 | 1 | 10 | | | |
| 21 | 8 | 0–512 | 6 | 1 | | | | |

[a] Basis of grading extent of thyroid damage: ±, small focal areas of inflammatory cells usually marked by a perivascular infiltrate; +, definite focal areas of mononuclear cell infiltrate which were principally perifollicular but follicular damage also noted; 2+, diffuse infiltration of thyroid follicles involving approximately 40% or less of thyroid tissue examined; 3+, infiltration and destruction of approximately 40–80% of thyroid tissue; and 4+, involvement of more than 80% of thyroid gland with some fibrotic deposition and adherence to adjacent muscle tissue.

matoxylin and eosin. Representative sections were examined for alterations in structure.

*Results.* Treatment of female Lewis rats with a single injection of RaTE incorporated in complete Freund adjuvant resulted in the appearance of thyroid lesions by 11 days after the sensitizing injection (Table I). It is significant that results of these experiments varied considerably with the preparation of RaTE employed. In one series of experiments using a single preparation of RaTE 4 of 6 animals showed lesions at 11 days, 7 of 22 had lesions at 14 days while 11 of 13 were positive at 17 days (Table I). On the other

TABLE II. The Effect of Pertussis Vaccine on Experimental Thyroiditis in Rats.

| Strain | Adjuvant | Duration (days) | Hemagglutination titer | | No. of animals with thyroid pathology | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | — | ± | + | 2+ | 3+ | 4+ |
| Lewis | CFA[a] | 3 | 0 | 0 | 2 | | | | | |
| | | 5 | 0 | 0 | 3 | | | | | |
| | | 10 | 0 | 0 | 3 | | | | | |
| | | 14 | 4 | 0–16 | 23 | | 1 | | | |
| | | 17 | 2 | 0–8 | 2 | | 1 | | | |
| | | 21 | 5 | 0–16 | 2 | 1 | | | | |
| Lewis | CFA + pertussis | 4 | 0 | 0 | 2 | | | | | |
| | | 7 | 4 | 0–64 | 64 | 11 | | | | |
| | | 10 | <2 | 0–4 | 4 | 2 | | | | |
| | | 14 | 3 | 0–128 | 3 | | 10 | 8 | 5 | 4 |
| | | 21 | 52 | 32–128 | | | 2 | 2 | 1 | |
| | | 28 | 32 | 8–64 | | | | 1 | 2 | |
| Fischer 344 | CFA + pertussis | 4 | 0 | 0 | 3 | | | | | |
| | | 7 | 4 | 0–8 | 4 | | | | | |
| | | 10 | 3 | 0–16 | 6 | | | | | |
| | | 14 | 3 | 0–16 | 33 | 6 | 4 | 2 | 2 | 2 |
| | | 21 | 8 | 2–64 | 1 | 2 | 1 | 1 | 2 | 1 |
| | | 28 | 37 | 32–64 | | | 1 | 1 | 3 | 4 |

[a] CFA = complete Freund adjuvant.

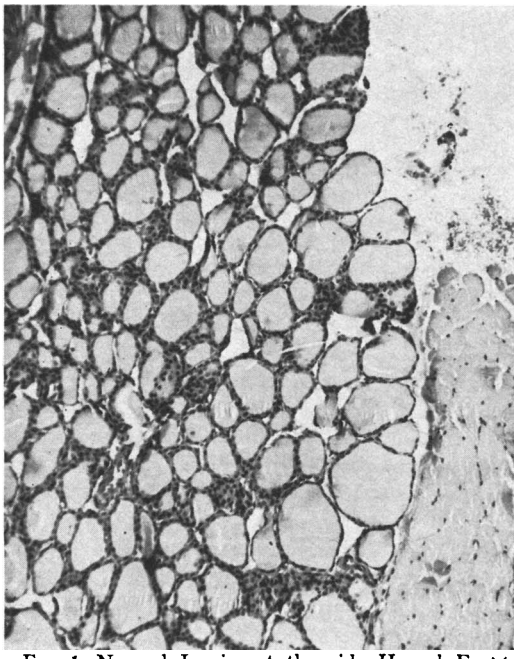

FIG. 1. Normal Lewis rat thyroid; H and E, × 100.

hand, in experiments where two other preparations of RaTE were used a total of only 3 of 30 animals had lesions of + or less, one each on days 14, 17, and 21 (Table II). Experiments with pertussis vaccine were performed using this latter group of extracts as well as several additional preparations which were not tested using complete Freund adjuvant alone.

When pertussis vaccine was administered in addition to the treatment used above, the lesions were consistently more severe. Whereas only one Lewis animal had lesions at 14 days using these RaTE extracts with Freund adjuvant alone, 12 of 13 animals had lesions at the same time when a simultaneous injection of pertussis was employed (Table II). Prolongation of the experiment did not result in increased severity of lesions although in the small number of animals available the lesions were all 2+ or greater at 28 days. Control animals receiving testis extract in place of thyroid extract had no thyroid pathology when examined 14 days after injection.

Fischer 344 rats receiving a similar injection first showed some evidence of thyroidal alterations at 14 days. However, whereas 90% of Lewis animals had thyroid pathology at this time, only 33% of the Fischer animals were similarly affected. Experiments without the simultaneous injection of pertussis vaccine were not conducted with this strain.

A single experiment was conducted with Lewis rats to determine the efficacy of pertussis vaccine as the sole adjuvant in inducing experimental thyroiditis. Sixteen rats were injected in the dorsum of each foot with a mixture of 0.2 ml of RaTE plus 0.5 ml of pertussis vaccine. Thus, complete Freund adjuvant was eliminated from this treatment. Four animals each were sacrificed on 7, 14, 21, and 28 days after treatment. There was no evidence of thyroidal alteration nor was circulating antibody directed against RaTE detectable at any of these times.

In both strains of rats early changes in thyroid histology consisted of perivascular infiltration of lymphocytic type cells (Figs. 1, 2). More striking changes involved interstiti-

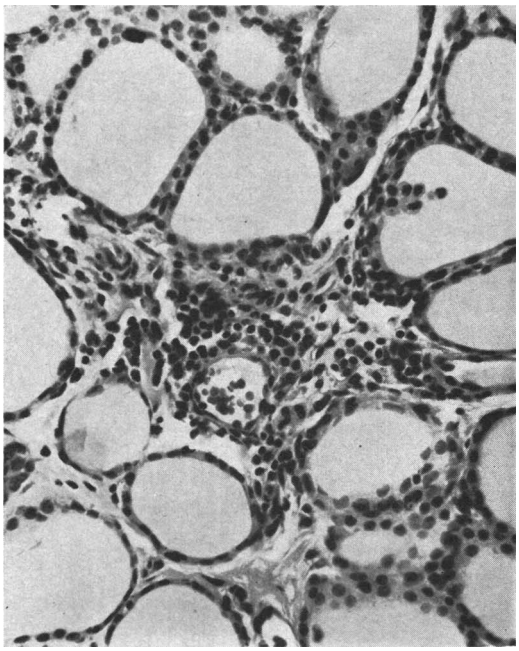

FIG. 2. Characteristic appearance of very early thyroid alteration in Lewis rats injected with RaTE Complete Freund adjuvant and pertussis vaccine marked by perivascular infiltration of mononuclear cells; H and E, ×450.

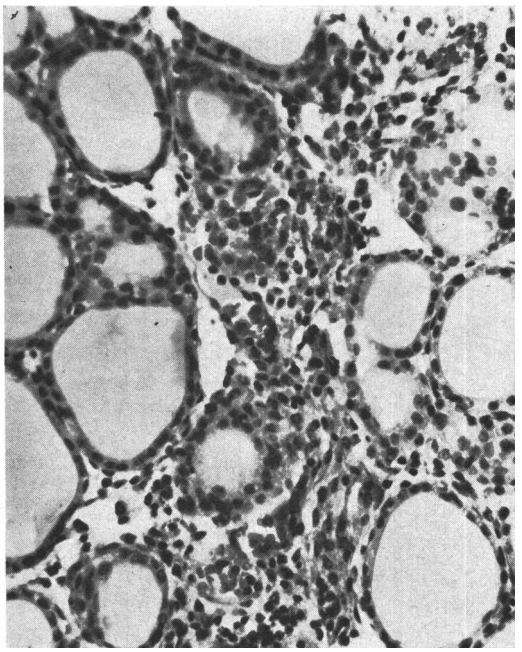

FIG. 3. Early thyroiditis (±) in Lewis rats characterized by interstitial lymphocytic infiltrate with no disruption of follicular architecture; H and E, ×450.

al infiltration by lymphoid cells (Fig. 3). Alterations varied in intensity from small focal areas of damage (Fig. 4) to involvement of the entire lobe (Fig. 5). In more severely involved glands complete distruption of follicular structure was observed in which colloid was entirely absent, the acini being filled with lymphoid- and macrophage-type cells. Fibrotic deposition also was sometimes noted (Fig. 5). The severe lesions in Fischer animals differed slightly from those in Lewis rats (Fig. 6). There appeared to be a tendency for the small lymphoid cells to remain grouped together in the thyroids of Fischer rats. In fact, an occasional formation resembling a germinal center could be seen.

*Discussion.* Jones and Roitt (7) using Wistar rats as well as Biörkland (8) using Sprague-Dawley animals obtained definitive thyroiditis following injection of an emulsion of rat thyroid extract (RaTE) in complete Freund adjuvant. Optimum results with single injections were obtained by Jones and Roitt using 2 mg of the antigen preparation. The present results indicate that simultaneous but separate injection of 100 billion killed *Bordetella pertussis* organisms together with the emulsion of RaTE and complete Freund adjuvant promotes the effectiveness of this antigen in inducing thyroid lesions. Using this regimen, thyroiditis could be elicited in a high percentage of treated Lewis rats by 14 days. Lewis animals began to develop thyroidal alterations earlier than Fischer 344 rats although once they appeared, the severity of lesions was comparable in both strains. Thyroiditis was not produced when pertussis vaccine was the sole adjuvant used.

Single injections of 6 mg of RaTE in complete Freund adjuvant elicited significant thyroid pathology in Lewis rats only when one particular preparation of RaTE was employed (Table I). This preparation of RaTE was used only for the series of experiments in Table I. There was some indication of reversability of thyroid disease in these animals as previously described by Jones and Roitt (7).

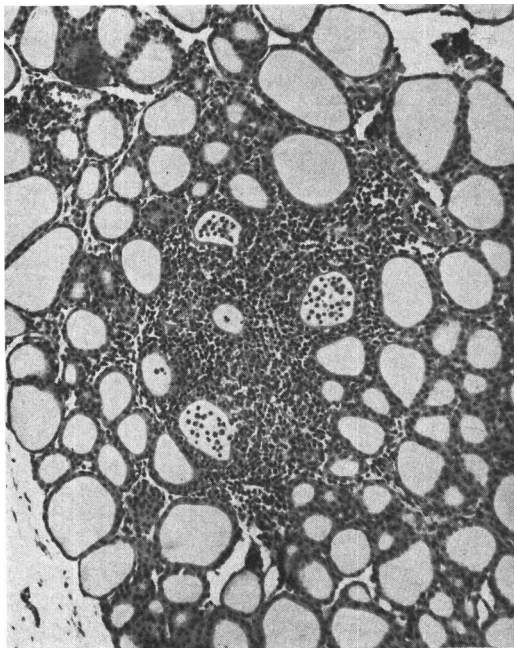

FIG. 4. Focal area of thyroid damage (+) in Lewis rats with both interstitial lymphocytic infiltration as well as some evidence of early follicular involvement; H and E, ×100.

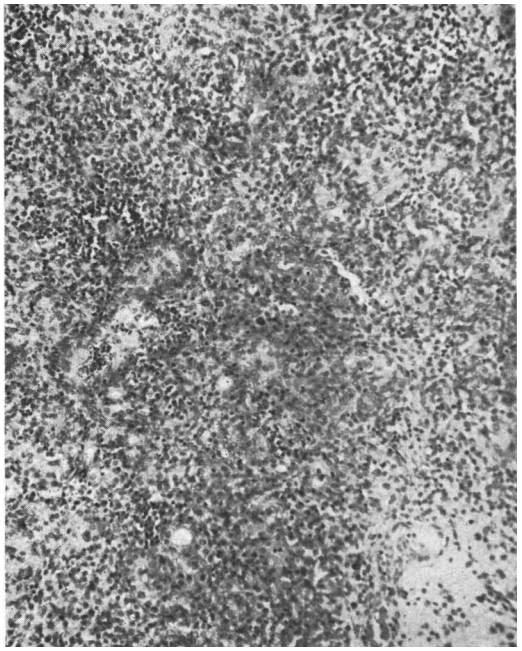

FIG. 5. Diffuse nature of severe thyroiditis (4+) in Lewis rats. Note the complete loss of follicular structure and evidence of fibrosis; H and E, × 100.

A similar regression was not seen during the limited duration of the present experiments in animals treated with pertussis.

Use of several other preparations of RaTE under identical conditions yielded much less consistent results. Other experiments with Lewis rats not reported here indicate that lesions comparable to those obtained with pertussis vaccine can be elicited using two or three spaced injections of RaTE in complete Freund adjuvant.

Although all thyroid extracts were procured from the same commercial source, all supposedly originating from random bred Wistar rats, marked differences in their ability to elicit thyroid lesions were noted. The enhanced antigenicity of one particular preparation could be due to the fact that the thyroids actually were from a different strain of rats. Alternatively, the antigenicity of this preparation could have been due to autolysis of the thyroid tissue at some time during its preparation. Previous work has shown that autolysis of tissue antigens may increase their immunogenicity (9).

The relative resistance of Lewis rats to induction of experimental thyroiditis following the method successfully employed in other species was unexpected. This strain of rats has been the animal of choice in studies on experimental allergic encephalomyelitis because of its high susceptibility to that autoimmune disease (10). The low incidence obtained may merely reflect a suboptimal matching of the origin of thyroid antigen with the immunized strain as indicated by the disparity of results with various extracts. In absolute terms, the Lewis animals were slightly more responsive to RaTE than were Fischer 344 rats. These results are similar to their order of susceptibility to allergic encephalomyelitis (10).

The mode of action of pertussis vaccine has not been clearly defined. In their extensive studies on its effect in experimental allergic encephalomeylitis, Levine and Wenk (11) found that pertussis vaccine retained the property of adjuvanticity whether it was incorporated in a Freund-type emulsion or ad-

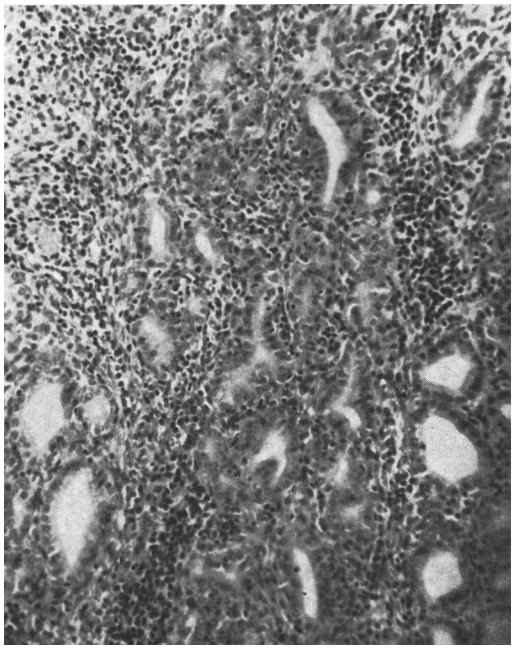

FIG. 6. Severe thyroiditis (4+) in Fischer 344 rat thyroid marked by extensive damage to follicular architecture. Note the tendency for aggregation of small lymphocytic cells; H and E, ×100.

ministered separately by various routes. However, the efficiency of the vaccine varied with the locus of injection. Best results were obtained when the vaccine was injected into an area draining to the same lymph nodes as the antigen.

In the case of allergic encephalomyelitis, the efficacy of pertussis vaccine is not associated with its endotoxin. Rather, it appears to be closely related to the histamine-sensitizing factor or a similar substance (12). Although pertussis vaccine causes splenic hyperplasia and peripheral leukocytosis in rats, neither of these activities appears to be important in its action as an adjuvant (11). Recent experiments on pertussis in mice indicate that the ability of this vaccine to increase phagocytosis by macrophages is also not related to its adjuvanticity (13). Several authors do indicate, however, that the adjuvant properties are connected to an antigenic component of the pertussis organism (13, 14).

Circulating antibodies, detectable by the tanned cell hemagglutination procedure, were elicited both in animals which received the RaTE only in complete Freund adjuvant as well as those also receiving the pertussis vaccine. Generally the level of antibody was not very high but an occasional animal did develop a titer of 128 or more. As in other studies of allergic thyroiditis (15, 16) there was no correlation of circulating antibody to RaTE with thyroid lesions. Animals having a high titer of antibody often had no histologic alterations while an occasional rat with no detectable antibody titer exhibited significant thyroid pathology.

*Summary.* Single injections of rat thyroid extract with complete Freund adjuvant were not consistent in eliciting significant thyroid pathology in Lewis rats. Identical treatment accompanied by a simultaneous but separate injection of 100 billion killed pertussis organisms was consistently effective in inducing marked thyroid changes at an earlier time. Lewis rats developed thyroid lesions approximately 1 week earlier than Fischer 344 rats receiving the same treatment. Pertussis vaccine also seemed to increase the level of circulating autoantibodies to thyroid.

We wish to acknowledge the competent technical assistance of Mrs. E. Kenney and Miss Roberta Lion. The pertussis vaccine was a generous gift of Dr. H. B. Devlin of Parke Davis Co., Detroit, Mich.

1. Lee, J. M. and Olitsky, P. K., Proc. Soc. Exptl. Biol. Med. **89**, 263 (1955).

2. Levine, S. and Wenk, E. J., Am. J. Pathol. **39**, 419 (1961).

3. Levine, S. and Wenk, E. J., Science. **146**, 1681 (1964).

4. Levine, S. and Wenk, E. J., Am. J. Pathol. **47**, 61 (1965).

5. Paterson, P. Y. and Drobish, D. G., J. Immunol. **101**, 11098 (1968).

6. Rose, N. R. and Witebsky, E., J. Immunol. **76**, 408 (1956).

7. Jones, H. E. H. and Roitt, I. M., Brit. J. Exptl. Pathol. **42**, 546 (1961).

8. Biörkland, A., Lab. Invest. **13**, 120 (1964).

9. Schwentker, F. F. and Rivers, T. M., J. Exptl. Med. **60**, 559 (1934).

10. Levine, S. and Wenk, E. J., Ann. N. Y. Acad. Sci. **122**, 209 (1965).

11. Levine, S. and Wenk, E. J., Am. J. Pathol. **50**, 465 (1967).

12. Levine, S., Wenk, E. J., Devlin, H. B., Pieroni, R. E., and Levine, L., J. Immunol. **197**, 363 (1966).

13. Dresser, D. W., Clin. Exptl. Immunol., in press.

14. Pieroni, R. E. and Levine, L., Nature. **211**, 1419 (1966).

15. Rose, N. R., Kite, J. H., and Doebbler, T. K., *in* "Mechanisms of Cell and Tissue Damage Produced by Immune Reactions" (P. Miescher and P. Grabar, eds.), p. 161. Benno Schwabe, Basel (1962).

16. Miescher, P., Gorstein, F., Benacerraf, B., and Gell, P. G. H., Proc. Soc. Exptl. Biol. Med. **107**, 12 (1961).

Received Sept. 3, 1968. P.S.E.B.M., 1969, Vol. 130.