Adjuvants for the Production of Autoimmune Thyroiditis in the Rat[1] (34437)

FRANK J. TWAROG,[2] ELLEN KENNEY, AND NOEL R. ROSE

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

Injection of aqueous heterologous thyroid extract has been shown to induce thyroid autoantibodies and thyroiditis in several species (1–3). On the other hand, experimental autoimmune thyroiditis cannot be produced by the simple injection of unaltered homologous thyroid extract (1, 4). Generally, in order to produce thyroiditis with homologous thyroid extract, an emulsion is prepared with complete Freund adjuvant (5). However, a variety of other procedures have been tried. Among the methods used were the coupling of thyroglobulin with diazonium derivatives of arsenilic and sulfanilic acid (6, 7), the injection of excess amounts of iodine in addition to thyroid extract (8) or the use of hypophysectomized animals (9). In addition, a variety of adjuvants have been studied (4, 10). Recently it has been found that the adjuvant employed is all-important for the production of thyroiditis in the rat (11, 12). Because of this finding we decided to study the effectiveness of different adjuvants and adjuvant combinations in the induction of thyroiditis in this species.

Materials and Methods. Animals. Female Lewis rats between the ages of 8 and 12 weeks were used. These animals were purchased from Simonsen Labs, Gilroy, Calif.

Antigen. A saline extract (pH 7.2) was prepared from Wistar rat thyroids (Pel-Freez Biologicals, Rogers, Ark.). The thyroid tissue was pulverized and this preparation was clarified by centrifugation at 69,000*g* for one-half hour. The protein concentration was adjusted to 30 mg/ml. Except in one experiment where the quantity of antigen used was varied, the dose of rat thyroid extract (RATE) used was 6 mg (0.2 ml).

Adjuvants. Complete Freund adjuvant (CFA) was prepared by adding 4 mg/ml killed *Mycobacterium tuberculosis* $H_{37}Ra$ to incomplete Freund adjuvant (Difco). Emulsions were prepared with either complete or incomplete Freund adjuvant by the dropwise addition of an equal volume of rat thyroid extract (RATE) to the oil phase while repeatedly drawing the material into a syringe through a long cannula and then expelling it. Except where noted specifically, the volume used was 0.2 ml of adjuvant plus 0.2 ml of antigen for each animal. This emulsion was administered intradermally into the foot pads and intramuscularly into the thighs except in a single series of experiments in which the intraperitoneal route was employed.

Pertussis vaccine (Parke Davis Co.) containing 200 billion killed organisms/ml was also used as an adjuvant. When this vaccine was used as the sole adjuvant, 0.5 ml was mixed with 0.3 ml RATE and then injected. When it was used as an adjunct to a Freund emulsion a volume of 0.5 ml of the vaccine was injected separately (12). This was distributed into the dorsum of the four feet.

The final adjuvant tested was alhydrogel. This is a commercially prepared adjuvant (Superfos, Copenhagen) which has properties similar to the alum preparations of Proom (13) when combined with an antigen. A volume of 0.2 ml alhydrogel was mixed with an equal volume of RATE prior to injection into the four footpads.

Antibody titration. The thyroid antibody

[1] Supported in part by U.S. Public Health Service Research Grants CA-05203 and CA-02357 from the National Cancer Institute, National Institutes of Health.

[2] Supported by U. S. Public Health Service Training Grant 5-T1-AI 130.

TABLE I. Production of Thyroiditis in Lewis Rats Using Various Adjuvants.

Adjuvant	Duration (days)	Hemagglutination titer (log_2) Mean	Range	Thyroid histology 0	±	+	2+	3+	4+	Index of pathology[a]
Incomplete Freund	7	0	0	6						0
	10	0.3	0–2	5						0
	14	2.3	0–4	6						0
	21	0	0	5						0
	28	0	0	5						0
	35	0	0	5						0
Complete Freund	10	0	0	3						0
	14	3.6	3–7	8	1		2			0.4
	21	2.7	0–9	8	2					0.1
	35	0	0–<2	1		1	3			1.4
Pertussis	7	0	0	4						0
	10	0	0	5						0
	14	0	0	4						0
	21	0	0	4						0
	28	0	0	9						0
	35	0	0	4						0
Alhydrogel	7	0	0	4						0
	10	0	0	4						0
	14	0	0	4						0
	21	0	0	5						0
	28	0	0	4						0
Incomplete Freund	7	0	0	4						0
+ pertussis	10	3	2–7	5						0
	14	0	0	2			1	1		1.2
	21	2	0–4	1		2	2	2		1.7
	28	0	0	1	2	2		1		1.0
	35	1.2	0–2	2		2				0.5
Complete Freund	7	0	0	7	1					0.1
+ pertussis	10	0.7	0–2	4	2					0.1
	14	2.2	0–7	2		2	3	2	1	1.8
	21	1.0	0–3			3	3	2		1.9
	28	3.7	3–6				1	3		2.7
	35	0.3	0–2	1		1	3			1.4
	49	0	0				2	3		2.6
	70	1.7	0–7				2	2		2.5
Complete Freund	7	0	0	9						0
+ pertussis[b]	10	0	0	13						0
	14	0.3	0–2	5			1			0.3
	21	0.1	0–2	3		2				0.4
	28	0.1	0–2	4	1					0.1
	35	1.1	0–4	3	1	1				0.3
Complete Freund	14	4.5	4–8	4						0
+ alhydrogel	21	6.5	6–7	1	3					0.4
	28	6.5	5–8	1	2	1				0.5

[a] Index of pathology was calculated by obtaining the mean of thyroid pathology using the following numerical values: 0 = 0; ± = 0.5; + = 1; 2+ = 2; 3+ = 3; 4+ = 4.

[b] Injections were given intraperitoneally.

titer of final bleedings was measured by tanned-cell hemagglutination (14). This titration was performed with microtitration equipment purchased from Cooke Engineering Co., Alexandria, Va.

Thyroid histology. Thyroid tissue was fixed in phosphate-buffered formalin solution. Representative sections were mounted and stained with hematoxylin and eosin. Thyroid pathology was graded as previously reported (12).

Results. The groups of animals in which either incomplete Freund adjuvant, pertussis vaccine, or alhydrogel was used as the sole adjuvant did not develop thyroidal alterations during the time periods studied (Table I). Except for some animals in the group treated with incomplete Freund adjuvant, no circulating thyroid antibodies were detectable.

When CFA was used alone the results were quite variable as previously reported (12). The development of thyroiditis was not predictable from one experiment to the next. However, thyroiditis as well as thyroid antibodies could be produced occasionally using this adjuvant alone. The significant index of pathology at 35 days (Table I) may indicate that this adjuvant takes a longer period to produce thyroiditis and perhaps the animals were observed too soon after immunization.

Combinations of adjuvants were generally more effective in inducing thyroiditis than any individual adjuvant tested. When aqueous pertussis vaccine was administered in addition to an emulsion of incomplete Freund adjuvant and RATE, thyroiditis was found to develop by 14 days.

The most consistently severe thyroiditis was elicited using an emulsion of CFA and RATE plus separate aqueous injection of pertussis vaccine. Reference to the index of pathology for this adjuvant combination readily shows that it was superior to all others tested. In addition, the groups of animals which were sacrificed at 49 and 70 days indicated that, at least with this treatment, the thyroid pathology is not quickly reversible. The same adjuvant combination injected intraperitoneally rather than into the foot pads and thighs was considerably less efficient.

Although alhydrogel did not elicit thyroid lesions when used alone, the simultaneous injection CFA did produce lesions in a fair number of rats. More importantly, this treatment elicited the highest titers of antibody found in any of the groups studied.

Since complete Freund adjuvant has been the classical adjuvant used in producing thyroiditis and since multiple injections of RATE and CFA had previously been shown to be effective in the rat (15) the present studies were extended to determine the efficacy of multiple injections of CFA and RATE. Two injections separated by a 1-week interval were very effective in producing thyroiditis especially at the 14-and 21-day intervals (Table II). However, the index of pathology for 28, 35, and 42 days indicates a decrease from the values obtained at 14 and 21 days. Thus, there appears to be a diminution of thyroiditis over the course of time, in contrast to the finding in those animals treated with CFA and pertussis (Table I). The use of three separate injections did not appear to be any more effective than two injections.

Finally, variation in the quantity of RATE injected was investigated. In this series of experiments rats were injected with an emulsion of CFA and various quantities of RATE plus the separate inoculation of pertussis vaccine (Table III). The results indicate that 6 mg of RATE, the amount used in all previous experiments, was the optimum dosage. The use of twice this dose did not improve the severity or incidence of thyroiditis.

Discussion. Previous studies on experimental thyroiditis have shown that except under unusual circumstances (6–9) some type of adjuvant must be employed to produce the disease with homologous thyroid extracts. Results of the present experiments confirm earlier studies of thyroiditis in other species and extend them to the rat.

An emulsion of RATE and incomplete Freund adjuvant, lacking *Mycobacterium tuberculosis,* was ineffective in inducing thyroiditis. Similarly, a mixture of aqueous pertussis vaccine and RATE was also ineffective. This situation differs from that in allergic encephalomyelitis where pertussis vaccine plus nervous-tissue extract produces a hy-

TABLE II. The Effect of Multiple Injections of Rat Thyroid Extract in Complete Freund Adjuvant.

Number of injections	Duration (days)[a]	Hemagglutination titer (log_2) Mean	Range	Thyroid histology 0	±	+	2+	3+	4+	Index of pathology
2[b]	14	5.6	4–7	2			1	1	1	1.8
	21	5.3	4–9			5		1		1.3
	28	7.0	4–10	1		1				0.5
	35	6.3	5–8		1	2				0.8
	42	—	—			3				1.0
3[c]	35	6.2	4–8	2		5	1			0.9

[a] Indicates the number of days after the initial injection.

[b] Injections were administered on days 0 and 7.

[c] Injections were administered on days 0, 9, and 23.

peracute form of the disease (16). Whereas each of these adjuvants used alone gave negative results, we found that a combination of the two was quite efficient in the production of thyroiditis. This combination has also been used in the production of allergic encephalomyelitis (17, 18). These findings confirm earlier studies which indicated that oil-in-water emulsions are not the sole requirement for production of thyroiditis. (5, 15).

Although complete Freund adjuvant (CFA) used alone induced thyroid lesions, the results of individual experiments were inconsistent, as previously reported (12). The addition of a separate injection of pertussis vaccine to this regimen, however, resulted in the most consistent production of severe thyroiditis of all adjuvants tested. Like Paterson and Drobish (11), we (12) have noted the marked enhancement of thyroiditis with the inclusion of pertussis vaccine. Even with this combination of adjuvants there was a striking difference noted when the route of immunization was altered. Foot-pad injections elicited severe thyroiditis but intraperitoneal injection of the same material proved to be a very poor method of inducing this disease.

These studies show that the efficacy of a CFA–RATE emulsion can be improved by the use of two or three spaced injections. The use of multiple injections of such an emulsion in rats has previously been advocated by Jones and Roitt (15). Even with the increased number of injections the results were not as satisfactory as with the pertussis–CFA combination. Another noteworthy difference between these two adjuvant regimens was the fact that even with repeated injections the incidence and severity of lesions appeared to

TABLE III. The Effect of Dose of Rat Thyroid Extract Used.[a]

Amount of RATE (mg)	Hemagglutination titer Mean	Range	Thyroid histology 0	±	+	2+	3+	4+	Index of pathology
0.3	0	0–0	4						0
3.0	1.0	0–4			1	3			1.7
6.0	1.2	0–4			1	4	1		2.0
12.0[b]	0.8	0–5		2	1	2	1		1.5

[a] The RATE was administered as an emulsion with complete Freund adjuvant; each rat also received a separate injection of 0.5 ml pertussis vaccine.

[b] The volume of complete Freund adjuvant used in this experiment was 0.4 ml; in all other experiments it was 0.2 ml.

decline with time. This confirms the earlier observations of Jones and Roitt (15) who also reported reversibility of thyroiditis in rats. However, with pertussis added to a single injection of CFA and RATE, lesions were present even 70 days after injection with no reduction of severity.

In studies of rabbit thyroiditis it has been shown that alum-precipitated homologous thyroid extract elicited antibody formation but no lesions. Addition of mycobacteria or a complete CFA emulsion to the alum preparation, however, elicited lesions as well as antibody (5, 10). Results of present investigations using the adjuvant alhydrogel, which is comparable to alum precipitation, are similar but not identical. Alhydrogel alone elicited neither lesions nor antibodies. Addition of CFA to the treatment elicited a slight degree of thyroiditis. Interestingly, this combination elicited the highest level of circulating thyroid antibodies found in our studies. Previous work also showed that the combination of alum and CFA elicited very high titers of antibody (10).

Finally, an experiment was performed to quantitate the amount of RATE required to induce thyroid lesions. A combination of CFA and pertussis adjuvants was employed. The amount of RATE used in all preceding experiments was 6 mg. Use of either half or twice this amount gave very similar results. However, 0.3 mg elicited neither antibody nor lesions. Jones and Roitt (15), with purified thyroglobulin rather than the crude saline extract used in these studies, found that they needed slightly less than this amount to induce thyroditis but the values were within the same range.

Summary. Use of either incomplete Freund, alhydrogel, or pertussis vaccine as adjuvants together with rat thyroid extract (RATE) elicited neither antibodies nor thyroiditis when injected into Lewis rats. A complete Freund adjuvant emulsion with RATE gave inconsistent results. Multiple, spaced injections of such an emulsion elicited a severe thyroid reaction. Combination of pertussis vaccine with either incomplete Freund adjuvant or alhydrogel elicited moderate thyroiditis. The most marked thyroiditis was obtained using a combination of CFA and RATE emulsion and a separate injection of pertussis vaccine.

The authors thank Mr. Steven Buck for his expert technical assistance. The pertussis vaccine was donated by Dr. H. B. Devlin of Parke, Davis & Co., Detroit, Mich. The alhydrogel was donated by Dr. Niels Neergaard, Superfos Export Co., Copenhagen, Denmark.

1. Terplan, K. L., Witebsky, E., Rose, N. R., Paine J. R., and Egan R. W., Am. J. Pathol. **36**, 213 (1960).

2. Weigle, W. O., and Nakamura, R. M., J. Immunol. **99**, 223 (1967).

3. Nakamura, R. M. and Weigle, W. O., Proc. Soc. Exptl. Biol. Med. **129**, 412 (1968).

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

5. Rose, N. R., Kite, J. H., Jr., Doebbler, T. K., Spier, R., Shelton, F. R., and Witebsky, E., Ann. N. Y. Acad. Sci. **124**, 201 (1965).

6. Weigle, W. O., J. Exptl. Med. **121**, 289 (1965).

7. Weigle, W. O., J. Exptl. Med. **122**, 1049 (1965).

8. Evans, T. C., Beirwaltes, W. H., and Nishigama, R. H., Endocrinology **84**, 641 (1969).

9. Godlowski, Z., Ann. Allergy **25**, 554 (1967).

10. Doebbler, T. K., and Rose, N. R., Federation Proc. **20**, 151 (1961).

11. Paterson, P. Y., and Drobish, D. G., J. Immunol. **101**, 1098 (1968).

12. Twarog, F. J., and Rose, N. R., Proc. Soc. Exptl. Biol. Med. **130**, 434 (1969).

13. Proom, H., J. Pathol. Bacteriol. **55**, 419 (1943).

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

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

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

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

18. Shaw, C., Alvord, F. C., Fahlberg, W. J., and Kies, M. W., J. Immunol. **92**, 28 (1969).

Received Sept. 11, 1969. P.S.E.B.M., 1970, Vol. 133.