

# A study of diverse humoral antibody systems following a single injection of Freund's incomplete adjuvant

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Initial clinical trials with repository therapy have been in progress at the Allergy Clinic of the Cincinnati General Hospital for several years. In the early trials, treatment consisted of an injection of ragweed emulsion given during the period from June 1 to June 15. Many patients who presented themselves for this therapy had symptoms from grass pollen as well as from ragweed pollen. Within a week after injection, it was noted that some patients who had been displaying seasonal symptoms (June grasses), reported a dramatic remission of their respiratory complaints.<sup>1</sup> Since a double-blind study of the effect of ragweed emulsion was in progress at that time, these initial observations were rather casually ascribed to a placebo effect. However, when similar results were noted during successive seasons of emulsion therapy, it was deemed advisable to study these effects by experimentation.

Several possible mechanisms seemed capable of accounting for the above observations: (1) a simple placebo effect; (2) a nonspecific effect of the type suggested by Pasheyev and colleagues<sup>2</sup>; (3) an effect resulting from the presence of cross-reactive allergens in ragweed and grass as indicated by Lidd and Farr<sup>3</sup>; and (4) an anamnestic response involving recall of diverse humoral systems in response to injections of an adjuvant.

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This report will concern itself with the results of studies designed to explore the role of a broad, nonspecific, and/or anamnestic effect of incomplete Freund's adjuvant upon human subjects. In addition, some corollary clinical data regarding the possible relationship of adjuvant to tissue damage will be presented.

## METHODS

### Patients

Forty-five patients were selected from the Allergy Clinic of the Cincinnati General Hospital. All patients had seasonal asthma and/or allergic rhinitis attributable to the pollens or molds. None of them had any significant unrelated diseases at the time of this study. Serum was obtained from each patient prior to treatment and 1 week and 4 weeks after injection. The sera were stored in the frozen state and shipped in dry ice to the various collaborators.

### Emulsion

The placebo emulsion used consisted of equal amounts of phosphate-buffered saline and a mixture composed of 35 per cent Arlacel A and 65 per cent mineral oil (Drakeol 6VR). A single injection consisting of 1 ml. of this emulsion was administered subcutaneously by means of a 25 gauge  $\frac{5}{8}$  inch needle.

### Immunologic studies

The determinations carried out and the appropriate methods are as follows:

1. Diphtheria antitoxin by the technique of Levine and associates.<sup>4\*</sup>
2. Tetanus antitoxin by the technique of Levine and associates.<sup>4\*</sup>
3. Heterophil by the technique of Davidsohn.<sup>5†</sup>
4. Cold agglutinins by the procedure outlined by Boyd.<sup>6†</sup>
5. Antistreptolysin O by the technique of Rantz and Randall.<sup>7†</sup>
6. Antithyroidal hemagglutinating antibody by the technique of Boyden.<sup>8†</sup>
7. Antigraass (June) hemagglutinating antibody by the technique of Boyden.<sup>8†</sup>
8. Antiragweed (short) hemagglutinating antibody by the technique of Boyden.<sup>8†</sup>
9. Poliomyelitis, strains I, II, and III, neutralizing antibody as described by Rogers and co-workers.<sup>9†</sup>
10. Mumps virus hemagglutinating antibody inhibition test by the technique of Robbins and associates.<sup>10†</sup>
11. Influenza virus A (Jap 305 strain) hemagglutinating antibody inhibition test by the method recommended by the Committee on Standard Serological Procedures in Influenza Studies.<sup>11†</sup>
12. *Herpes simplex* hemagglutinating antibody inhibition test as described by Dascomb and colleagues.<sup>12†</sup>

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\*These tests were performed by Mr. Leo Levine at the Division of Biologic Laboratories, Massachusetts Department of Public Health, Forest Hills, Boston, Mass.

†These tests were performed under the direction of Dr. Saul Malkiel of the Children's Cancer Research Foundation, Boston, Mass.

‡These tests were performed under the direction of Dr. Francis C. Cadigan, Jr., of the Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D. C.

13. Complement-fixing antibodies to calf thymus nucleoprotein,<sup>13</sup> calf thymus,<sup>14</sup> and human liver saline extracts.<sup>14\*</sup>
14. VDRL test for syphilis by the technique of Harris and associates.<sup>15†</sup>
15. Latex fixation tests (gross, euglobulin, and inhibition techniques) for rheumatoid factor by the techniques of Singer<sup>16</sup> and Hall.<sup>17†</sup>
16. Bentonite flocculation test for deoxyribonucleoprotein (lupus factor) by the technique of Bozicevich and co-workers.<sup>18†</sup>
17. Cephalin flocculation by the technique of Hanger.<sup>19†</sup>
18. Total protein and A/G ratio by the technique of Kingsley.<sup>20, 21†</sup>

## RESULTS

Of the forty-five patients who received the single placebo injection, thirty-one returned for special studies. In this group weekly and monthly serum specimens were obtained from all but four patients who failed to return for the monthly visit. The VDRL tests for syphilis were negative for all patients before and after treatment. In every instance cephalin flocculation was negative before and after treatment. There was no significant alteration in the level of the total protein or A/G ratio in any of the tested samples. The battery of latex techniques for rheumatoid factor revealed no abnormalities. Lupus factor determined by the DNA bentonite flocculation test did not appear in any sera in this investigation. Presumptive evidence for the absence of antinuclear antibodies was also obtained by uniformly negative complement-fixing antibodies against calf thymus nucleoprotein.

Table I is a list of quantitative data on the levels of diphtheria antitoxin, tetanus antitoxin, serial dilutions of heterophil antibodies and cold agglutinins, anti-streptolysin O, and antigriass and antiragweed hemagglutination antibodies. It is apparent that diphtheria and tetanus antibodies were not appreciably affected by the injection of incomplete Freund's adjuvant. Low titers of heterophil antibodies were found, but in all instances these values were not significant and did not become elevated after treatment. Almost identical results were observed with regard to cold agglutinating activity. Antistreptolysin titers were essentially unchanged before and after treatment. Hemagglutinating antigriass and antiragweed antibodies were not affected to any appreciable degree by the injection of adjuvant. In the griass system one patient showed a twofold rise in antigriass hemagglutinating antibody, from a titer of 1:80 to one of 1:160. A fourfold and a fivefold increase in titer were obtained in two other patients, but there was no detectable pretreatment activity in these cases. These results are isolated findings and can hardly be considered significant. Relative changes in antiragweed antibody levels are even less noteworthy.

Table II summarizes the data concerning viral antibodies. It may be stated that no general rising titer pattern is discernible. There are a few examples of

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\*These tests were performed by Miss Lois A. Simonton, Department of Serology, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D. C.

†These tests were performed under the direction of Dr. I. Leonard Bernstein at the Cincinnati General Hospital, Cincinnati, Ohio.

**Table I.** Bacterial, pollen, and nonspecific serum antibodies before and after a single injection of placebo emulsion

| Serum antibody                                       | Before<br>(No. of<br>patients) | After<br>(No. of patients) |    |    |   |           | No change<br>(No. of<br>patients) |
|------------------------------------------------------|--------------------------------|----------------------------|----|----|---|-----------|-----------------------------------|
| <i>Diphtheria antitoxin</i><br>( $\mu\text{g/ml.}$ ) |                                | 32                         | 16 | 8  | 4 | 2 or less |                                   |
| 32                                                   | 4                              | 3                          | 1  | 0  | 0 | 0         |                                   |
| 16                                                   | 1                              | 0                          | 1  | 0  | 0 | 0         |                                   |
| 8                                                    | 4                              | 0                          | 1  | 2  | 1 | 0         |                                   |
| 4                                                    | 0                              | 0                          | 0  | 0  | 0 | 0         |                                   |
| 2 or less                                            | 22                             | 0                          | 0  | 0  | 0 | 22        |                                   |
| <i>Tetanus antitoxin</i><br>( $\mu\text{g/ml.}$ )    |                                | 64                         | 32 | 16 | 8 | 4         | 2 or less                         |
| 64                                                   | 2                              | 1                          | 1  | 0  | 0 | 0         | 0                                 |
| 32                                                   | 3                              | 0                          | 1  | 2  | 0 | 0         | 0                                 |
| 16                                                   | 2                              | 0                          | 0  | 1  | 1 | 0         | 0                                 |
| 8                                                    | 3                              | 0                          | 0  | 0  | 3 | 0         | 0                                 |
| 4                                                    | 0                              | 0                          | 0  | 0  | 0 | 0         | 0                                 |
| 2 or less                                            | 21                             | 0                          | 0  | 0  | 0 | 0         | 21                                |
| <i>Anti June grass</i><br>(titer*)                   |                                | Tube increase†             |    |    |   |           |                                   |
|                                                      |                                | 1                          | 2  | 3  | 4 | 5         |                                   |
| 0                                                    | 24                             | 0                          | 0  | 0  | 1 | 1         | 22                                |
| 5                                                    | 1                              | 0                          | 0  | 0  | 0 | 0         | 1                                 |
| 20                                                   | 4                              | 0                          | 0  | 0  | 0 | 0         | 4                                 |
| 40                                                   | 1                              | 0                          | 0  | 0  | 0 | 0         | 1                                 |
| 80                                                   | 1                              | 1                          | 0  | 0  | 0 | 0         | 0                                 |
| <i>Antiragweed</i><br>(titer*)                       |                                | Tube increase†             |    |    |   |           |                                   |
|                                                      |                                | 1                          | 2  | 3  | 4 |           |                                   |
| 0                                                    | 19                             | 2                          | 0  | 1  | 1 |           | 15                                |
| 10                                                   | 4                              | 1                          | 0  | 0  | 0 |           | 3                                 |
| 20                                                   | 4                              | 0                          | 0  | 0  | 0 |           | 4                                 |
| 40                                                   | 4                              | 0                          | 0  | 0  | 0 |           | 4                                 |
| <i>Anti streptolysin O</i><br>(Todd units)           |                                | Tube increase†             |    |    |   |           |                                   |
|                                                      |                                | 1                          |    |    |   |           |                                   |
| 10                                                   | 7                              |                            |    |    | 1 |           | 6                                 |
| 50                                                   | 11                             |                            |    |    | 3 |           | 8                                 |
| 100                                                  | 4                              |                            |    |    | 1 |           | 3                                 |
| 150                                                  | 3                              |                            |    |    | 0 |           | 3                                 |
| 200                                                  | 3                              |                            |    |    | 1 |           | 2                                 |
| 250                                                  | 1                              |                            |    |    | 0 |           | 1                                 |
| 333+                                                 | 2                              |                            |    |    | 0 |           | 2                                 |
| <i>Heterophil</i><br>(titer*)                        |                                | Tube increase†             |    |    |   |           |                                   |
|                                                      |                                | 1                          |    |    |   |           |                                   |
| 0                                                    | 22                             |                            |    |    | 0 |           | 22                                |
| 7                                                    | 3                              |                            |    |    | 0 |           | 3                                 |
| 14                                                   | 1                              |                            |    |    | 0 |           | 1                                 |
| 28                                                   | 5                              |                            |    |    | 1 |           | 4                                 |
| <i>Cold agglutinins</i><br>(titer*)                  |                                | Tube increase†             |    |    |   |           |                                   |
|                                                      |                                | 1                          |    |    |   |           |                                   |
| 0                                                    | 27                             |                            |    |    | 0 |           | 27                                |
| 7                                                    | 1                              |                            |    |    | 0 |           | 1                                 |
| 14                                                   | 2                              |                            |    |    | 0 |           | 2                                 |
| 28                                                   | 1                              |                            |    |    | 0 |           | 1                                 |

\*Reciprocal of titer.

†This denotes increase in tube dilution titers from the base-line titer.

**Table II.** Viral serum antibodies before and after a single injection of placebo emulsion

| <i>Serum antibody</i>               | <i>Before</i><br>(No. of patients) | <i>After</i><br>(No. of patients) |          |          | <i>No change</i><br>(No. of patients) |
|-------------------------------------|------------------------------------|-----------------------------------|----------|----------|---------------------------------------|
| <i>Poliomyelitis, type I</i>        |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> | <i>3</i> |                                       |
| Less than 10                        | 4                                  | 1                                 | 0        | 0        | 3                                     |
| 10                                  | 2                                  | 0                                 | 0        | 1        | 1                                     |
| 20                                  | 4                                  | 0                                 | 0        | 0        | 4                                     |
| 40                                  | 2                                  | 1                                 | 0        | 0        | 1                                     |
| 80                                  | 5                                  | 1                                 | 1        | 0        | 3                                     |
| 160                                 | 4                                  | 0                                 | 0        | 0        | 4                                     |
| 320                                 | 5                                  | 1                                 | 0        | 0        | 4                                     |
| 640 or more                         | 2                                  | 0                                 | 0        | 0        | 2                                     |
| <i>Poliomyelitis, type II</i>       |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> | <i>3</i> |                                       |
| Less than 10                        | 7                                  | 0                                 | 2        | 1        | 4                                     |
| 10                                  | 3                                  | 0                                 | 0        | 0        | 3                                     |
| 20                                  | 1                                  | 1                                 | 0        | 0        | 0                                     |
| 40                                  | 7                                  | 2                                 | 0        | 0        | 5                                     |
| 80                                  | 7                                  | 1                                 | 0        | 0        | 6                                     |
| 160                                 | 2                                  | 0                                 | 0        | 0        | 2                                     |
| 320                                 | 1                                  | 0                                 | 0        | 0        | 1                                     |
| 640 or more                         | 0                                  | 0                                 | 0        | 0        | 0                                     |
| <i>Poliomyelitis, type III</i>      |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> | <i>3</i> | <i>4</i>                              |
| Less than 10                        | 5                                  | 0                                 | 0        | 1        | 0                                     |
| 10                                  | 3                                  | 0                                 | 1        | 0        | 1                                     |
| 20                                  | 6                                  | 3                                 | 1        | 0        | 0                                     |
| 40                                  | 1                                  | 1                                 | 0        | 0        | 0                                     |
| 80                                  | 4                                  | 1                                 | 1        | 0        | 0                                     |
| 160                                 | 2                                  | 0                                 | 0        | 0        | 0                                     |
| 320                                 | 4                                  | 3                                 | 0        | 0        | 0                                     |
| 640 or more                         | 3                                  | 0                                 | 0        | 0        | 0                                     |
| <i>Mumps</i>                        |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> | <i>3</i> |                                       |
| Less than 10                        | 3                                  | 0                                 | 0        | 0        | 3                                     |
| 10                                  | 3                                  | 3                                 | 0        | 0        | 0                                     |
| 20                                  | 13                                 | 3                                 | 1        | 0        | 9                                     |
| 40                                  | 8                                  | 1                                 | 1        | 0        | 6                                     |
| 80                                  | 1                                  | 0                                 | 0        | 0        | 1                                     |
| <i>Influenza A (Jap 305 strain)</i> |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> | <i>3</i> |                                       |
| 40                                  | 1                                  | 0                                 | 1        | 0        | 0                                     |
| 80                                  | 3                                  | 0                                 | 3        | 0        | 0                                     |
| 160                                 | 5                                  | 2                                 | 1        | 1        | 1                                     |
| 320                                 | 5                                  | 2                                 | 1        | 0        | 2                                     |
| 640                                 | 5                                  | 1                                 | 0        | 0        | 4                                     |
| 1,280                               | 6                                  | 0                                 | 0        | 0        | 6                                     |
| <i>Herpes simplex</i>               |                                    |                                   |          |          |                                       |
| <i>(titer*)</i>                     |                                    | <i>Tube increase†</i>             |          |          |                                       |
|                                     |                                    | <i>1</i>                          | <i>2</i> |          |                                       |
| Less than 8                         | 4                                  | 0                                 | 0        |          | 4                                     |
| 8                                   | 7                                  | 2                                 | 0        |          | 5                                     |
| 16                                  | 9                                  | 1                                 | 1        |          | 7                                     |
| 32                                  | 8                                  | 1                                 | 0        |          | 7                                     |

\*Reciprocal of titer.

†This denotes increase in tube dilution titers from the base-line titer.

**Table III.** *Tissue antibodies before and after a single injection of placebo emulsion*

| <i>Tissue antibody</i>                                    | <i>Before<br/>(No. of patients)</i> | <i>After<br/>(No. of patients)</i> | <i>No change<br/>(No. of patients)</i> |
|-----------------------------------------------------------|-------------------------------------|------------------------------------|----------------------------------------|
| <i>Anti-human thyroid (hemagglutinating)</i>              |                                     |                                    |                                        |
| <i>(titer*)</i>                                           |                                     | <i>Tube increase†<br/>1</i>        |                                        |
| 0                                                         | 24                                  | 1                                  | 23                                     |
| 5                                                         | 1                                   | 0                                  | 1                                      |
| 20                                                        | 1                                   | 0                                  | 1                                      |
| 40                                                        | 0                                   | 0                                  | 0                                      |
| 80                                                        | 2                                   | 0                                  | 2                                      |
| 160                                                       | 1                                   | 0                                  | 1                                      |
| 320+                                                      | 2                                   | 0                                  | 2                                      |
| <i>Anti-human liver (complement fixing)</i>               |                                     |                                    |                                        |
| <i>(titer*)</i>                                           |                                     | <i>Tube increase†<br/>1</i>        |                                        |
| 0                                                         | 27                                  | 1                                  | 26                                     |
| 3                                                         | 0                                   | 0                                  | 0                                      |
| 9                                                         | 1                                   | 0                                  | 1                                      |
| <i>Anti-calf thymus (complement fixing)</i>               |                                     |                                    |                                        |
| <i>(titer*)</i>                                           |                                     | <i>Tube increase†<br/>1 2</i>      |                                        |
| 0                                                         | 25                                  | 2 1                                | 22                                     |
| 3                                                         | 2                                   | 0 0                                | 2                                      |
| 9                                                         | 1                                   | 0 0                                | 1                                      |
| <i>Anti-calf thymus nucleoprotein (complement fixing)</i> |                                     |                                    |                                        |
| <i>(titer*)</i>                                           |                                     | <i>Tube increase†<br/>1</i>        |                                        |
| 0                                                         | 26                                  | 1                                  | 25                                     |
| 3                                                         | 2                                   | 0                                  | 2                                      |

\*Reciprocal of titer.  
†This denotes increase in tube dilution titers from the base-line titer.

increased titers after the emulsion injection, but these are too sporadic to be important.

Table III is a compilation of the results of tissue antibodies studied in this project. It is curious that some antithyroidal hemagglutinating activity was present in seven patients, none of whom had any clinical evidence of thyroid disease. However, the titer of these antibodies is generally low, suggesting an entirely nonspecific mechanism. In one patient the antithyroidal hemagglutinating level rose from 0 to 1:5 after the repository injection. Low titers of complement-fixing antibodies against a saline extract of human liver were observed in one patient, but no change was noted after the injection.

**DISCUSSION**

The data in this pilot study seem to indicate that injections of incomplete Freund's adjuvant do not produce any significant alterations in a wide variety of humoral antibody systems. This conclusion, of course, applies only to the time covered in this study, i.e., a 1 month interval following the injection. In

addition, we observed no detectable changes in liver function, and there was no *de novo* appearance of antitissue antibodies. These results are of interest to those who are concerned with applications of adjuvant therapy in humans, inasmuch as there have been considerable speculation and discussion about the possible long-range effects of oil droplets which, after being phagocytized, could conceivably migrate to distant sites in the reticuloendothelial system.

Our interest was also focused upon the uniformly negative results from the various rheumatoid factors observed in this investigation. Perhaps a brief digression on why these tests were included in this study is germane at this point. In two patients a peculiar type of migratory polyarthritis had been observed about 1 week after emulsion depot treatment. Positive latex fixation tests were obtained from these patients. Arthropathy subsided spontaneously after a period of 6 to 8 weeks. Both of these patients had diabetes mellitus. One was of the juvenile type and the other of a moderately severe type of adult diabetes accompanied by diabetic changes in both retinas. It may be that appearance of joint changes in these patients was purely coincidental, but a possible association between this type of arthropathy and adjuvant arthritis experimentally produced in rats was suggested.<sup>22</sup> Thus it appeared worthwhile for us to pursue rheumatoid factor studies in greater detail in this particular group of subjects. It was interesting to note that no apparent serum rheumatoid activity was elicited by the emulsion that could be determined by some of the more sensitive rheumatoid tests currently available.

In conclusion, we interpret our results to indicate that a diffuse anamnestic response is not to be expected after the injection of incomplete Freund's adjuvant, at least as far as humoral antibodies are concerned.

It is interesting that five patients in this study reported subjective improvement 7 to 10 days after the placebo injection. For this small group, at least, two explanations are tenable: (1) the effects of suggestive therapy (placebo effect) and (2) the nonspecific therapy effect of Pashayev and associates.<sup>2</sup> According to these authors, mineral oil and certain refined products derived from oil possess biologic properties which tend to suppress many forms of allergic processes. Although no confirmation of this work has been forthcoming as yet, it is interesting to note that several reports by Jankovic<sup>23, 24</sup> have shown that delayed skin reactivity to bovine serum albumin, human gamma globulin, and experimental allergic thyroiditis were all markedly suppressed in guinea pigs by prior injections of the animals with adjuvant alone. The possible effect of emulsion on delayed hypersensitivity was not included in the present study.

The occurrence of cross-reactive groupings in grass and ragweed pollens can be suggested as an explanation for the clinical effects observed in this investigation of placebo emulsion. From their experimental results on the primary interaction between radioiodinated ragweed pollen and antibodies in human sera, Lidd and Farr<sup>3</sup> noted that ragweed pollen extract probably contains antigenic groups similar to antigens derived from other plant sources, including grass pollen. It is possible that some of the beneficial results obtained previously in this clinic have been from this type of phenomenon.

## SUMMARY

A wide spectrum of humoral antibody systems has been evaluated in thirty-one patients before and after they received a single placebo injection of saline solution emulsified with an Arlacel-Drakeol mixture. Certain liver function parameters and antitissue antibodies were also investigated at the same time. There were no significant alterations in viral, bacterial, or pollen antibody titers following this injection. Tests for rheumatoid activity, lupus factor, and antitissue antibodies were negative, and liver function was not affected by the subcutaneous deposition of emulsion. These results indicate that the clinical effects of oily repository materials are not mediated by a nonspecific anamnestic type of response. They also suggest that oily emulsions are apparently not hazardous to certain vital organs, at least within a one-month period after administration.

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