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IMMUNOLOGICAL STUDIES OF POLLINOSIS

III. Fluctuations in Antibody-titer of Normal Individuals Subcutaneously and Intravenously injected with Pollen-extract over Protracted Periods[1]

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In 1937 Cooke, Loveless and Stull (1) reported that normal individuals respond to pollen-inoculations by forming immune antibody.

This antibody can inhibit the urticarial response of sensitized skin to pollen by interfering with the pollen-reagin reaction. (In the absence of immune antibody, skin inoculated with reagin-bearing serum from a hay-fever patient promptly develops erythema and a wheal when pollen-antigen is introduced.) Subsequent studies (2) showed that the cutaneous response fails to occur in the presence of immune serum because pollen is bound and inactivated by the immune antibody. This antibody was found to differ from reagin not only in its greater avidity for pollen-antigen and in its ability to unite with antigen without irritating surrounding tissue, but also in its rapid disappearance from skin into which it had been injected and in its capacity to resist temperatures that inactivate reagin. The latter observation made it possible to eliminate reagin and thereafter to demonstrate separately the existence of immune antibody in the sera of pollen-sensitive patients who had received pollen-inoculations. Such patients acquire immune antibody not only in their sera but also in their tissues (3).

The observations about to be described were made in conjunction with studies on pollen-immune sera. In an attempt to produce antisera of unusually high titer, various inoculation-procedures were employed. This report will show the influence of these variations in procedure on three normal volunteers who received inoculations of ragweed-pollen antigen (1) in clear solution as compared with the alum-precipitated form, (2) subcutaneously as compared with intravenously and (3) as a "secondary" stimulus following various rest-periods.

It is known that immunization of animals is best achieved through intravenous introduction of the antigen, providing the latter is particulate. This has been deomonstrated for bacterial suspensions by Schultz and Swift (4), Julianelle (5), Freund and Opie (6), and for alum-toxoid by Freund and Bonanto (7). Plain nonparticulate toxoid, on the other hand, appears to be more effective by the subcutaneous route (8, 9), although Watanabe (10) concluded that hens respond to toxin more readily and more persistently when they are inoculated intravenously. The influence of a rest-period during immunization upon the response of an animal to additional antigen is illustrated by Freund and Bonanto (7). Finally, Beard, Finkelstein and Beard (11) have demonstrated a comparable enhancement in the response of human subjects to revaccination with equine encephalomyelitic virus.

[1] Presented at the Twenty-eighth Annual Meeting of the American Association of Immunologists at Chicago, Illinois, on April 17th, 1941.

MATERIAL AND METHODS

The three volunteers were in their early twenties, gave no personal or familial history of hypersensitiveness, and failed to respond to all appropriate tests done in the skin, eye and with the serum. Two of the subjects received low-ragweed pollen extracted in alkaline saline solution, whereas the third was given an initial series of the same extract after alum [$(Al_2SO_4)_3K_2SO_4 \cdot 24H_2O$] had been added to yield a final concentration of 2 per cent. The pollen-solutions used for inoculations and for serological titrations were standardized according to their content of protein-nitrogen (1.0 mg of phosphotungstic-acid-precipitable nitrogen = 100,000 P.N. units). The initial course consisted of increasing amounts of extract given twice weekly under the skin for about four months and resembled the therapy of hay-fever except that greater dosage was involved. After the primary course of inoculations and a subsequent "rest-period", two of the subjects were given additional antigen in an attempt to elicit an accelerated response. The immunological response to such "secondary" stimuli was tested on numerous occasions in one volunteer who was reinoculated after rest-periods which varied in length from 12 days to over a year. Although the antigen was customarily introduced subcutaneously, on two occasions it was given intravenously.

The serological response of the three volunteers was studied at intervals, a total of 104 specimens of serum being titrated. The immune-antibody content of each specimen was assessed by its antigen-inactivating power (2). The latter was revealed by adding increasing concentrations of antigen to a constant volume of serum, the resultant mixtures being tested for free antigen in uniformly sensitized cutaneous sites. The standard site prepared in numerous nonsensitive subjects throughout the present experiment required antigen in the ratio of 100 units per ml of serum for its desensitization. When the antigen was mixed with an equal volume of immune serum, more concentrated antigen was needed to effect desensitization, 20,000-unit extract being required in some instances. Such desensitization-experiments, carried out to determine the antigen-binding power of a given immune serum, were repeated in as many as 10 different individuals, the endpoints being averaged in order to minimize experimental error.

EXPERIMENTAL

The typical procedure and results pertaining to one volunteer will now be presented in some detail.

The subject, H, received alkaline saline extract of low-ragweed pollen from mid-April to mid-August of 1938. The first dose in this initial series of subcutaneous inoculations was 1000 P.M. units (about 100 times that customarily given an untreated hypersensitive patient). The dosage was rapidly increased up to a maximum of 175,000 units which is about 20 times the largest dose ordinarily administered a pollen-sensitive patient. Two months of such treatment produced, as graph 1 shows, slight but definite antigen-binding capacity in the serum, for on June 6th 200-unit antigen was required to desensitize the standard site used in titrating all sera whereas 100-unit antigen sufficed when normal serum was mixed with the antigen. The dose was then gradually advanced to 105,000 units and

the titer meanwhile rose to 2000 (August 8). On August 31, twelve days after the maximal dose of 175,000 units had beem given, and again one week later, the titer was found unchanged. (The highest titer hitherto observed in the author's pollen-sensitive patients is 2000.)

About 6 months elapsed between the initial course of injections and a second series of 3 subcutaneous inoculations. Nineteen days prior to the beginning of the second course, the titer had been 1000. On January 25, 1939, 1000 units were administered subcutaneously. The next day 10,000 units were given and on the third day 139,000, making a total of 150,000 units. Three days later the titer was found unchanged at 1000. By February 3, however, it had reached 10,000. By March 10, the titer was 5000. Eight days later two injections, totalling 150,000 units were given one day apart. When tested after 9 days, the titer had not changed but on April 14, 25 days after the last inoculation, the volunteer's serum neutralized antigen in the ratio of 7500 units per ml of serum.

Two attempts were subsequently made, one in June and another in August, to induce a titer of more than 10,000 by means of 150,000 units of antigen given in 3 injections, but they were unsuccessful as graph 1 shows. The titer then gradually fell during a rest-period of five months to 1500 where it remained for another month. A single inoculation of 150,000 units was then given on February 27, 1940. A rise of titer to 20,000 occurred within two weeks. A second inoculation of the same amount, given two weeks after the first, failed to increase the antigen-binding factor in the serum beyond this point. The titer was maintained fairly close to this maximum for two weeks after the second injection but had definitely diminished after three months, being found at 6000 on June 21, 1940. It declined further during the next six months, reaching 3000 by December, 1940, and remained at this level during the ensuing four months.

The next step was to study the relative effectiveness and the safety of introducing extract directly into the circulation.

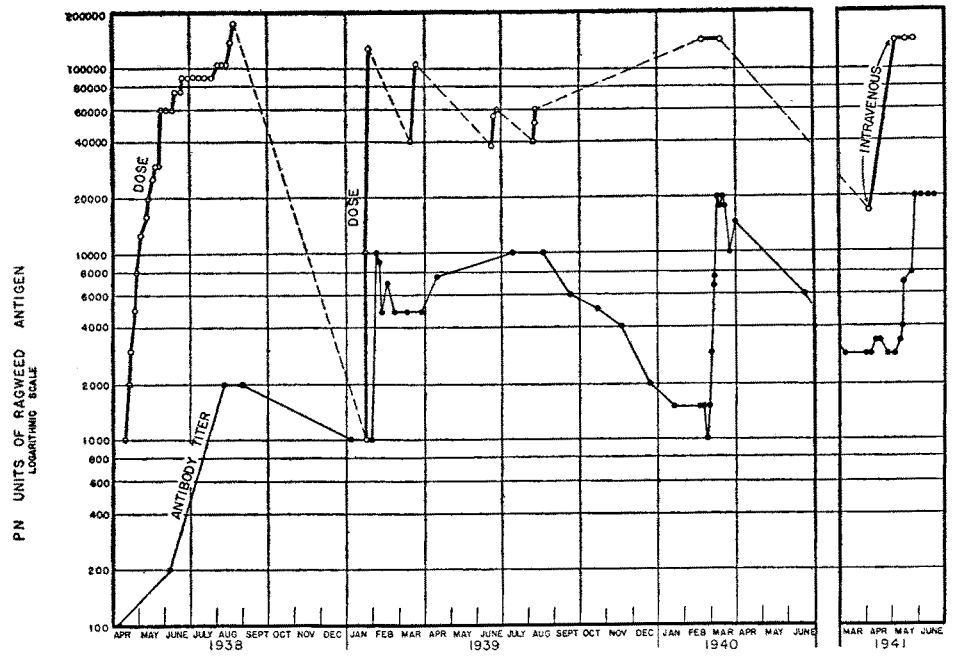
Accordingly, on April 3, 1941, after a rest-period of over 12 months, 100 units were given intravenously. As no immediate objective or subjective reaction occurred in the subject, 1500 and then 15,000 units were given at 15-minute intervals with no untoward effects. Titrations carried out on specimens obtained in 1, 3, 6 and 16½ hours, respectively, and on 12 other occasions after the intravenous injections revealed only insignificant changes in antibody-titer during 4 weeks.

On May 2, 1941, one month after the first intravenous injections, 150,000 units were administered intravenously in 400 ml. of physiological saline solution over a period of 40 minutes, without observable effect upon the recipient. Serum obtained three times during the first 24 hours showed no change in antigen-binding capacity, and only a slight increase in titer was detectable on the 7th day. On the 11th and 14th days, the titer was 7000, approximately twice the preinjection level.

Since it seemed probable that no further rise would occur spontaneously, 150,000 units were given subcutaneously on May 16, 1941. The titer rose gradually within 12 days to 20,000. A final subcutaneous dose of the same amount given on the 12th day failed to cause any further increase in antigen-binding capacity within 9 additional days of observation.

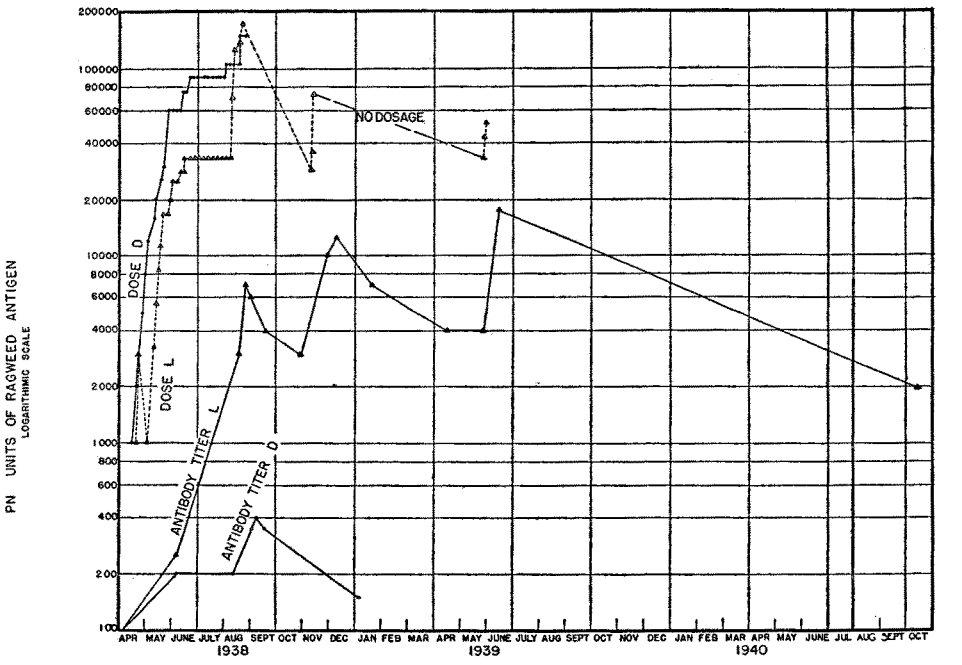
The type of immunological response shown by the other two volunteers who were inoculated with ragweed-extract is indicated in graph 2. The reactions did not differ significantly from those described above except that the volunteer, D, produced relatively little humoral antibody despite the fact that her injections paralleled in time and exceeded in total dosage those of the initial series given to the male volunteer, H.

The third recipient of ragweed-extract was L, who received alum-precipitated extract during the first four months of his initial course. The maximal dose of alum-extract received by him was 34,000 units, less than half the 90,000-unit dose received by each of the



RAGWEED ANTIGEN DOSE AND RESULTANT ANTIBODY TITER

GRAPH 1 shows antibody-titer that resulted from a primary series of pollen inoculations and from subsequent reinoculations with 9 "secondary" doses of 150,000 units each, given in from one to three injections.



RAGWEED ANTIGEN DOSE AND RESULTANT ANTIBODY TITER

GRAPH 2

other subjects at the corresponding time. After 12 semi-weekly inoculations of 34,000 units each, L was given 70,000 units of plain extract without noticeable reaction. His next three injections were increased rapidly to a maximum of 175,000 units of the alkaline saline solution. As shown in graph 2, the blood of L developed a maximal neutralizing capacity which was over three times that of the first subject, albeit his total dose of alum-precipitated (602,300) plus plain extract (507,500) was decidedly less than the total quantity given to H (2,162,500). After three months of freedom from injection, L was given 137,750 units of alum-extract in three doses, a day apart. His titer rose from a preinjection level of 3000 to 12,500 by the 26th day after injection. During the next 6 months, his titer gradually fell to 4000. A total of 127,000 units of plain extract, administered in three doses between May 26 and 31, raised the titer to 17,500 within 13 days. When a serological study was done on a specimen of blood taken nearly a year and a half later, L still showed definite evidence of immunity, his serum neutralizing 2000 units.

None of the 104 specimens of serum obtained from the three subjects during the entire experiment possessed skin-sensitizing reagins for ragweed-pollen.

LOCAL REACTIONS

The first injection caused no observable local reaction in any of the 3 volunteers. Local cutanous reactions followed subsequent inoculations of pollen extract.

A slight erythema coming on after 10 and 27 hours in H and D, respectively, occurred following the second inoculation of alkaline saline extract. The third inoculation caused a slight redness after 2 hours which lasted 1½ hour and was associated with slight itching in the case of D, but the third and the fourth inoculations evoked no reaction in H. D showed no detectable response to the fourth and fifth injections. Slight local reactions then occurred in both subjects after shorter intervals and persisted for longer periods after successive doses until the 9th injection, which was followed by erythema within 10 minutes. The reaction endured 24 hours in H, and 60 hours in D. Thereafter the reactions in D occurred a few hours after injection and consisted of erythema and induration, 2 to 3 cm in diameter. They lasted for a few hours to a day, and were associated with a slight tendency toward pruritus and tenderness. In the case of H, the 10th, 11th and 12th injections caused erythema and induration which were observed within ten minutes to one hour and which endured for an increasingly long time (at most for 60 hours). Thereafter the onset of the reaction varied irregularly from a few minutes to 31 hours and was accompanied by local itching for a few minutes after inoculation. The areas of induration ranged in size from 2 cm to 7 cm in their largest diameters.

The local responses of L to alum-precipitated extract were more delayed and prolonged than were those just described. By the 9th injection, however, as with the unprecipitated extract, an immediate erythema occurred which lasted for two days. After this, the precipitated extract elicited reactions only after some hours. There was no associated itching at any time but a slight or moderate amount of induration was palpable. Near the end of the initial course, L was given plain instead of alum-precipitated extract. No unusual reaction was observed, although the first dose of plain extract was more than twice that which had been administered in the precipitated form four days earlier. Nine hours after final inoculation of 175,000 units of plain extract, however, L developed marked induration and a headache which lasted for 40 hours.

Reinoculation, preceded by various rest-periods, was associated with delayed local induration and tenderness which were slight in most instances. However, 150,000 units given to H on May 16, 1941, caused marked induration and tenderness which lasted 36 hours. Injections of plain extract administered to L on May 26, 27, and 31, 1939, resulted promptly in marked local urticaria and in delayed local induration of marked degree, associated with slight axillary tenderness on the side of the inoculation. The day following the inoculation, intracutaneous tests with 10,000-unit extract elicited a moderate reaction, whereas more

dilute antigen caused no response. This acquired cutaneous sensitivity was transient and was not associated with conjunctival sensitivity.

Hence, with rare exceptions, the observable reactions of the nonsensitive individuals to pollen-inoculations were slight and local. In two instances reactions to reinoculation were more marked than those elicited during the initial courses. None of the subjects developed any sign of clinical hypersensitiveness to pollen during four years' observation.

DISCUSSION

Although the number of experimental observations are too few to permit definite conclusions to be drawn, it is interesting to note that during the initial, 4-month series of inoculations, the person receiving alum-precipitated antigen showed the most marked humoral response, despite the fact that the total dose given him was about half that of either of the individuals receiving plain extract. (L received 602,300 units of alum-extract and 507,500 units of plain extract, a total of 1,109,800 units; the others, 2,162,500 and 2,372,500 units of plain extract, respectively.) Graph 2 illustrates the antibody-response noted after precipitated extract and after plain antigen. Whether the difference in response is attributable to alum-precipitation of the antigen, or to individual difference in immune response or to both, is not now known. Both the alum-extract and the plain extract, when given after a rest-period, elicited a definite enhancement in titer (Cases L and H). Individual difference in immunological response was clearly demonstrated in the instances of D and H. In both cases plain antigen was given. Graphs 1 and 2 illustrate how D, though receiving 210,000 units more antigen than H during the primary series, nevertheless produced humoral antibody only one-fifth as high in titer.

Alum-extract usually produced more delayed and more prolonged local reactions than did the plain extract. During their first series of injections all three subjects showed a lessening delay in local response until, by the 9th injection, the reaction was detected within 15 minutes. Thereafter, the response varied in the time of its appearance from a few minutes to hours.

None of the 104 sera obtained from the three individuals during the 4 year experimental period revealed a capacity for skin-sensitization. However, the possibility of reagin-formation during the early weeks cannot be excluded since no sera were obtained after the onset of the experiment until 12 inoculations had been given. Intracutaneous tests done at various times failed to reveal any acquired sensitiveness of the skin to ragweed-pollen, with the exception of a slight, transient reaction observed in the subject who received alum-extract. No clinical sensitiveness has been detected during four years observation. Further study is required to explain the fluctuations in local response. Since pollen-extract is a complex solution containing numerous antigens, it is possible that these local reactions may depend on immune bodies different from the humoral antibodies referred to in this article.

Reinoculations with a large dose of antigen given in one or several injections induced a marked enhancement in humoral antibody-response. The extent of

the enhanced, secondary response varied with the interval between the eliciting stimulus and the last preceding injection. One subject, H, who was tested numerous times with reinjections after rest-periods of variable duration showed an optimal response when the antigen was given subcutaneously after an interval of six months or more. The neutralizing power of his serum after reinoculation was 20,000 units of ragweed-extract per ml of serum—ten times greater than any observed by the writer to date in pollen-sensitive patients following an initial series of inoculations. When the reinoculation was given this nonsensitive individual by the intravenous route, even the full dose of 150,000 units produced only slightly more than a twofold increase over a previously low titer. Two weeks later 150,000 units given subcutaneously elicited the maximal response, bringing the titer to 20,000. Repetition of this subcutaneous dosage in 12 days failed further to enhance the serum antibody-content.

The recall-phenomenon was also elicited in L on two occasions. Precipitated antigen, given subcutaneously 3 months after the primary series had been completed, elicited a fourfold increase in titer to 12,500 whereas a somewhat smaller amount of plain extract, given subcutaneously after a rest-period of 6 months, brought the titer to 17,500.

In all instances, the antibody-titer resulting from reinoculation far surpassed that from the primary series, despite the fact that a much larger total dose was involved in the latter. An attempt is now being made to evoke an accelerated response in pollen-sensitive patients. It is hoped thereby to enhance their immunity and shorten the time required for effective therapy following a primary series of inoculations.

The finding that the intravenous route was less effective than the subcutaneous as a means of immunizing man against clear solutions of antigen is in keeping with the previous observations already mentioned.

Failure to observe a negative phase following the administration of antigen is not surprising. The lowest titer observed previous to reinoculation in the present series of cases was 1500. Theoretically 100 ml of such antiserum would serve to neutralize the largest dose given (150,000 units). Assuming that all the antigen reached the blood-stream, the amount of antibody neutralized per ml of the entire blood-volume would still be smaller than the experimental error of titration, so that any tendency toward a negative phase would be effectively masked.

CONCLUSIONS

1. Of three nonsensitive volunteers who were given inoculations of pollen extract twice weekly for four months, the one receiving alum-precipitated extract showed the highest titer of thermostable neutralizing antibody in his serum.

2. Two of them receiving alkaline saline extract of low-ragweed pollen in similar dosage differed markedly in the degree of their immune response.

3. Reinoculation, following the initial series of injections and a "rest-period" of 3 months or more, produced a striking enhancement in immune response when the reinoculation was given subcutaneously. The same dose of clear pollen-extract, administered by the intravenous route, was far less effective.

4. Immediate and delayed local reactions to subcutaneous inoculations of plain or alum-precipitated extract were usually mild. No generalized reaction was observed, with the exception of headache in one instance.

5. Evidence of acquired hypersensitiveness was shown by only one of the 3 volunteers. This individual had received alum-precipitated extract and developed a temporary and slight cutaneous sensitiveness without associated clinical allergy.

6. None of the 104 specimens of serum obtained from the 3 inoculated subjects possessed skin-sensitizing antibody (reagin) to ragweed-pollen.

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