

Demonstration of human reagin in the monkey

II. In vitro passive sensitization of monkey ileum with sera of untreated atopic patients

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Ever since the discovery of skin-sensitizing antibody (reagin) in human serum by Prausnitz and Küstner in 1921,¹ numerous attempts have been made to demonstrate this substance by other techniques. The weight of evidence supports the view that several different types of antibodies are found in human allergic sera. The tanned-cell or bis-diazotized benzidine (B.D.B.) hemagglutination techniques probably determine both precipitating and skin-sensitizing antibodies in the sera of treated and untreated patients.² The radioimmuno-electrophoretic technique,³ the primary binding of I¹³¹-labeled ragweed pollen antigen,⁴ and the coprecipitation method of antihuman globulin and I¹³¹-labeled antigen⁵ probably all demonstrate the presence of precipitating and reaginic antibodies in atopic sera.

The estimation of histamine release from actively or passively sensitized leukocytes after the addition of the appropriate antigen^{6, 7} may be a way of specifically detecting reagins. However, the results are not always reproducible because of the relatively small amounts of histamine evolved by the reaction. Degranulation of basophils occurs when antigen and allergic sera are added to the cells; this procedure has been described⁸ but is not readily standardized.

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Only a relatively small percentage of allergic sera seems to be able to induce this cellular transformation. Recently, Ridges and Augustin⁹ reported an in vitro test for atopic reagins by double-layer leukocyte agglutination. The main proof of the reaginic nature of the antibodies was the absence of agglutination after the sera had been heated for seven hours at 56° C.

In addition to these various in vitro experimental methods, Layton and his co-workers¹⁰ have reported that serum from atopic individuals can passively sensitize the skin of the monkey. This work has been confirmed and elaborated upon in our laboratory.¹¹

Dale, in 1913,¹² and, recently, Halpern and co-workers¹³ have demonstrated that the guinea pig ileum can be sensitized in vitro with antibodies of the precipitating type. An anaphylactic contraction occurs when the appropriate antigen is added to the sensitized muscle strip suspended in a water bath (Schultz-Dale apparatus). The conditions and factors involved in this sensitization have been carefully studied,¹⁴ and it was found that this reaction could be inhibited with the addition of nonspecific gamma globulins.¹⁵

To our knowledge, it has never been demonstrated that sera from untreated atopic individuals could regularly sensitize the guinea pig ileum either in vitro or by systemic administration. It therefore occurred to us that if monkey skin could fix human skin-sensitizing antibodies, perhaps the smooth muscle of the monkey ileum could do the same. Hence, using certain modifications of the Halpern method, we sought to determine if this would be a feasible and sensitive technique to measure antibodies in sera of allergic patients.

MATERIALS AND METHODS

Antisera

Sera were collected from atopic individuals sensitive to one or more allergens (ragweed, *Alternaria*, caddis fly, grasses, house dust, and feathers) who had had no previous specific injection therapy. These sera were stored at 4° C. until they were studied. Eight sera from patients of the atopic group, not clinically sensitive and with negative intradermal skin tests to ragweed pollen extract, were used as controls in testing the ragweed antigen-antibody system. Sera were also obtained* from ten European patients who were not sensitive to and who had never been exposed to ragweed. These sera were employed as additional controls. Antiragweed rabbit sera were prepared as previously described.¹⁶

Antigens

The antigens used in these experiments included extracts of defatted short ragweed pollen and *Alternaria tenuis* (both prepared in Coca's solution without phenol) as well as saline extracts of caddis fly, feathers, house dust, and grass pollen.

Anaphylaxis in vitro

Normal monkeys (*Macaca mulatta*, *Macaca irus*, and *Saimiri sciurea*) were utilized in these studies. The monkeys were given an intravenous injection of

*Obtained through the courtesy of Dr. Claude Langlois, Hospital Broussais, Paris, France.

Nembutal sodium and then bled out. About 20 cm. of ileum, just proximal to the ileocecal valve, was immediately removed and the lumen carefully rinsed with Tyrode's solution. The tissue was stored at 4° C. in a large amount of the same physiologic medium until used. It was noted that these muscle tissues would still react well 48 hours after removal from the animal.

The ileum from the guinea pig was obtained after first bleeding the animal from the heart and then sacrificing it with a blow on the head.

The muscle strips were cut into sections 1.0 to 1.5 cm. long and placed in individual test tubes containing 9 ml. of Tyrode's solution at 37° C. To each tube was added 1.0 ml. of varying dilutions of serum for in vitro sensitization and the mixture kept at 37° C. for one hour. The muscle strips were then washed several times and suspended in a 10 ml. capacity muscle chamber. Air was bubbled through the Tyrode's solution in this bath. One end of the muscle was attached by a silk thread to a transducer which transmitted the isometric contraction through a preamplifier to an ink-writing polygraph (Grass Model A). The other end of the muscle strip was fixed to a wire hook attached to the glass tube which brought air into the muscle chamber. The base line was established with a 1.0 Gm. weight, and the sensitivity remained unchanged throughout the experiment.

This recording system avoided the resistance and inertia of the writing lever and thus allowed a much higher amplification, thereby producing a greater degree of sensitivity than that obtained with the classical Schultz-Dale equipment.

Prior to challenging a sensitized strip, a piece of nonsensitized monkey ileum was tested with varying dilutions of histamine in order to calibrate the smooth muscle response. It was found that high concentrations of ragweed and *Alternaria* extracts also caused contractions of unsensitized monkey or guinea pig ileum.

Consequently, a dilution of these antigens which did not produce this type of nonspecific reaction was utilized to challenge the sensitized strip.

With the use of this sensitized muscle strip, the histamine sensitivity was rechecked before the antigen was added. After the anaphylactic contraction, the same amount of specific antigen was added to the bath until there was no further response. The sensitivity to histamine was again checked at the end of the experiment.

RESULTS

Sensitization of monkey ileum

Each of the twenty-eight sera from patients known to be sensitive to ragweed, *Alternaria*, and other allergens was used to sensitize strips of monkey ileum. A positive anaphylactic contraction occurred when the specific antigen was added to the muscle chamber containing the sensitized strip of muscle. The maximum concentration of antigen used was that which would not produce a nonspecific contraction of an unsensitized portion of the same ileum. The degree of response was measured in terms of histamine equivalents. After the initial contraction, the muscle was washed several times and the same antigen again added to the bath. The second contraction was considerably less than the first.

Table I. "Blind test" *in vitro* sensitization of monkey and guinea pig ileum with sera of atopic individuals

Patient	Ragweed sensitivity	Ileum response	
		Monkey	Guinea pig
Ra.	0	0	+
Wi.	0	0	0
Ko.	0	0	0
Ge.	0	0	+
De.	0	0	0
Da.	0	0	0
Ber.	0	0	++
So.	0	+	+++
Be.	++++*	++++†	++++
La.	+++	++	++++
As.	+++	++	+
Ad.	++	+	++++
Br.	++	++	+

*Relative degree of sensitivity as measured by direct intradermal skin tests.

†Relative degree of sensitivity as determined by contraction following addition of ragweed pollen extract.

Eventually, after this procedure was repeated several times, the ileum no longer responded to additions of the same antigen but would still respond to histamine.

When the serum of a patient known to be allergic to more than one antigen was used to sensitize the muscle strip, after complete desensitization (lack of response to allergen following several successive contractions), the addition of the second antigen still produced an undiminished anaphylactic muscle reaction. Several sera were tested from patients who were known to be sensitive to many antigens, and each antigen produced a specific response.

Specificity of the reaction

Ten sera from European patients who had no contact with ragweed were used to sensitize strips of monkey ileum. They were each challenged with ragweed pollen antigen. There was a very minimal response with only one of these sera; the others completely failed to react. As a positive control, a strip of this same ileum was sensitized with a serum from a ragweed-sensitive patient and challenged with the same concentration of ragweed extract. A strongly positive contraction was demonstrated.

As a further control, to test the validity and specificity of the reaction of the ragweed system, thirteen sera from atopic patients were studied. These sera were supplied to one of us (P. G.) and identified by code number only; he did not know which of them were from ragweed-sensitive patients. Eight of these sera were from patients sensitive to other allergens who had negative intradermal skin tests and a negative clinical history for sensitivity to ragweed. The remaining five sera were from the positive reactors. They were tested by sensitizing both the monkey and guinea pig ileum.

As will be noted in Table I, only one of the sera from individuals not sensitive to ragweed* gave a minimal response when the monkey ileum was used, whereas

*On subsequent retesting of this patient, she developed a marked delayed reaction to an intradermal injection of ragweed pollen extract (300 pnu per milliliter).

all five of the ragweed-sensitive sera were able to sensitize strips of monkey ileum. The degree of contraction was roughly parallel to the intradermal reaction of the patient. When guinea pig ileum was used, however, four of the eight ragweed-negative sera showed response. (Each of these four sera had negative tanned-cell hemagglutination titers.) All five of the ragweed-positive sera produced positive contractions when ragweed extract was added.

The following experiments were performed to give additional evidence of the specificity of the responses. Monkey ileum was passively sensitized with varying dilutions of sera of twelve patients. In most instances, the type and degree of the sensitivity of the patients were unknown to the investigator performing the test. Each strip was then challenged with a constant amount of ragweed or *Alternaria* extract. The minimal amount (highest dilution) of serum which still produced a contraction following challenge was considered the end point of the reaction. The results of these experiments are shown in Table II.

The sera of patients who had a negative intradermal skin test to a 1:10 dilution of a 3 per cent extract of *Alternaria* usually failed to sensitize monkey ileum. A few of these sera, when used in higher concentrations (1:3), could slightly sensitize the muscle strip. In some instances, the minimal amount of serum which could sensitize the muscle was not determined due to lack of sufficient tissue at the time of the particular experiment. It must be noted that dilutions of human serum up to 1:5,000 could still sensitize the monkey ileum and produce a strong contraction when ragweed antigen was added.

An example of the high degree of sensitivity of this reaction is illustrated in Fig. 1. A portion of an actual tracing is reduplicated. The upper part of the figure demonstrates the muscular contraction response to various concentrations of histamine and the nonspecific contraction of the unsensitized muscle strip with different amounts of ragweed and *Alternaria* extract. It will be noted that a 1:500 dilution of the 15 per cent ragweed extract (200 pnu per milliliter) gave a nonspecific contraction, whereas a 1:2,000 dilution (50 pnu per milliliter)

Table II. *Comparative sensitivity of intradermal skin tests and contraction of sensitized monkey ileum*

Patient	Intradermal skin tests*		Sensitized monkey ileum†	
	Ragweed	<i>Alternaria</i>	Ragweed	<i>Alternaria</i>
Pra.	+++	+++	1:5,000	> 1:1,000
Rog.	++++	++	1:5,000	1:1,000
Ueb.	++++	+	> 1:5,000	1:1,000
Fei.	++++	++	1:5,000	1:1,000
Got.	++	+++	1:5,000	> 1:1,000
Tei.	+++++	+	> 1:5,000	1:100
Hun.	+	+	1:1,000	1:100
Ron.	++++	+	1:1,000	1:50
Chr.	++	0	> 1:1,000	1:10
Vel.	++++	0	> 1:1,000	0
Gia.	++	0	> 1:1,000	0

*Relative degree of intradermal sensitivity.

†Highest dilution of antiserum which still sensitized the monkey ileum as evidenced by contraction upon subsequent addition of the respective antigen. Dilutions tested were 1:10, 1:50, 1:100, 1:500, 1:1,000, and 1:5,000. > 1:1,000 indicates that a positive response was obtained at this dilution, but higher dilutions could not be tested.

was negative. Likewise a 1:50 dilution of a 3 per cent extract of *Alternaria* did not produce a contraction with the unsensitized strip. These latter dilutions of the antigens were then used to challenge the sensitized muscle strips. In the lower graph are depicted the contractions produced by these antigens when added to ileum previously sensitized with varying dilutions of serum. It will be noted that the ragweed still produced a contraction with a strip sensitized with a 1:5,000 dilution of serum, whereas the *Alternaria* extract no longer elicited a response with this dilution of antiserum.

Studies on thermolability

Sera from previously untreated ragweed-sensitive patients and an antiragweed rabbit serum were heated at 56° C. for varying periods of time ranging from 15 minutes to 6 hours. These, along with unheated samples, were then employed to sensitize both monkey and guinea pig ileum. Each of the strips was challenged with the same concentration of ragweed antigen. In Fig. 2, one notes the effect of the heating of one of the human ragweed sera upon its ability to sensitize the monkey and guinea pig ileum. The top tracing demonstrates the response of the monkey ileum, and, at 2 hours heating at 56° C., the serum was unable to sensitize the strip as indicated by the almost complete lack of response to ragweed challenge. On the other hand, the lower graph records the response of the guinea pig ileum with the same serum and under the same conditions. It will be observed that there was little change in response even after the serum was heated for six hours.

Heating the antiragweed rabbit serum did not affect its ability to sensitize

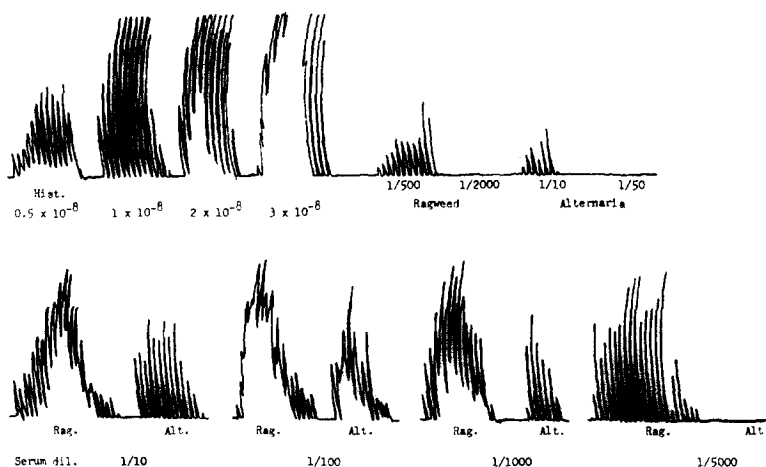


Fig. 1

Quantitative sensitization of monkey ileum with serum of patient allergic to both ragweed and *Alternaria*. Upper tracing: Contractions of unsensitized monkey ileum to various dilutions of histamine (expressed in micrograms per milliliter), of 15 per cent extract of short ragweed pollen extract, and of 3 per cent extract of *Alternaria*. Lower tracing: Contractions of strips of monkey ileum sensitized with varying dilutions of serum from a patient sensitive to both ragweed and *Alternaria* and challenged with the 1/2,000 dilution of ragweed extract or the 1/50 dilution of *Alternaria*. Both tracings read from left to right.

either the monkey or guinea pig ileum. A summary of the effects of heating upon sensitization of several sera from ragweed-sensitive patients is graphically shown in Fig. 3.

Quantitative studies

Varying dilutions of fifteen allergic sera were used to sensitize both the monkey and guinea pig ileum. Tanned-cell hemagglutination titers of all these

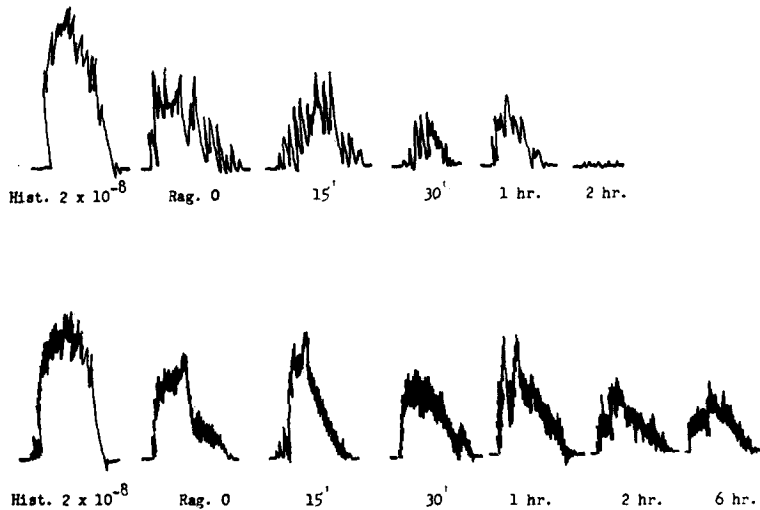


Fig. 2

Effect of heating serum from an untreated ragweed-sensitive patient on in vitro sensitization of monkey and guinea pig ileum. Upper tracing: Record of the effect of challenge with ragweed pollen extract on strips of monkey ileum sensitized by unheated patient's serum or serum heated at 56° C. for 15 minutes, 30 minutes, 1 hour, and 2 hours. Lower tracing: Reactions with guinea pig ileum heated under the same conditions.

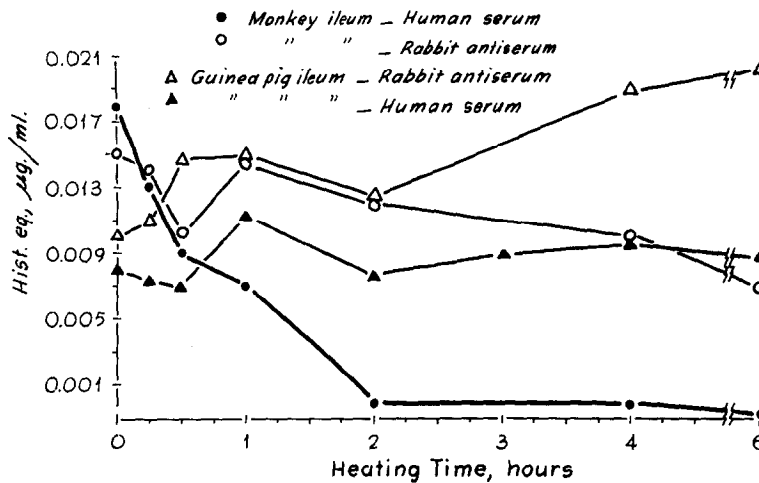


Fig. 3

Effect of heating human and rabbit antisera on their ability to sensitize monkey and guinea pig ileum.

sera were determined. As will be noted in Table III, there did not appear to be any relationship between these two types of tests. Several sera with relatively low or even negative hemagglutination titers still were able to sensitize the monkey ileum in relatively high dilutions. It should be pointed out that, in general, those sera which had low or negative hemagglutination titers had nega-

Table III. *Sera from ragweed-sensitive patients—Comparison of tanned-cell hemagglutination and in vitro sensitization of monkey and guinea pig ileum*

Patient	Hemagglutination titer*	Sensitized ileum	
		Monkey†	Guinea pig
Chr.	Negative	1:1,000	1:10
Gott.	Negative	1:5,000	1:10
Hun.	Negative	1:1,000	Negative
Ebe.	Negative	1:5,000	1:10
Gaw.	Negative	1:10,000	1:10
Sim.	1:20	1:1,000	1:10
Chip.	1:20	1:1,000	Negative
Beg.	1:80	1:6,000	Negative
Vel.	1:160	1:1,000	1:500
Rog.	1:160	1:5,000	1:500
Hoak.	1:160	1:1,000	1:100
Caci.	1:640	1:1,000	N.D.‡
Ron.	1:640	1:1,000	1:100
Tei.	1:640	> 1:5,000	N.D.
Gla.	1:640	> 1:1,000	N.D.
Pra.	1:640	1:5,000	1:500
Hus.	1:1,280	1:5,000	1:10
Vel.	1:1,280	> 1:5,000	1:500
Fei.	1:1,280	1:5,000	1:100

*Highest dilution of serum that produced a positive hemagglutination.

†Highest dilution of serum which could still sensitize an ileum (monkey or guinea pig) and produce a response on subsequent challenge with ragweed extract.

‡N.D., Not done—insufficient serum.

Table IV. *Sera from ragweed-sensitive patients—Comparison of skin sensitizing antibody in human recipients to in vitro sensitization of monkey and guinea pig ileum*

Patient	Reagin titer*	Sensitized ileum†	
		Monkey	Guinea pig
Ca.	3	1:1,000	N.D.‡
Chr.	3	1:1,000	1:10
Hun.	3	1:1,000	1:10
Glas.	9	> 1:1,000	N.D.
Ron.	27	1:1,000	1:100
Tei.	27	> 1:5,000	N.D.
Hoak.	27	1:1,000	1:100
Fei.	81	1:5,000	1:100
Pra.	81	1:5,000	1:500
Rog.	243	1:5,000	1:500
Vel.	243	1:1,000	1:500
Hus.	729	1:5,000	1:10

*Reciprocal of highest dilution of serum which produced positive passive transfer reactions in human skin.

†Highest dilution of serum which sensitized ileum of monkey and guinea pig in vitro.

‡N.D., Not done.

tive or low end points in sensitization of guinea pig ileum. It was also observed that the end point is 10 to 100 times greater with the use of the monkey ileum as compared to that with guinea pig ileum.

The skin-sensitizing antibody titers of twelve of these sera were determined by injecting threefold dilutions into the skin of each of two nonatopic recipients. These results were compared to those obtained when in vitro sensitization of monkey and guinea pig ileum was employed and are listed in Table IV.

Eight of these sera were also measured by injecting serial dilutions into the skin of a *Macaca irus* or *Macaca mulatta* monkey and then challenging with an intravenous injection of ragweed extract and Evans blue dye.¹¹ The results compared with those of in vitro sensitization of monkey and guinea pig ileum are shown in Table V.

Table V. Sera from ragweed sensitive patients—Comparison of skin-sensitizing antibody in monkey recipients to in vitro sensitization of monkey and guinea pig ileum

Patient	Reagin titer*	Sensitized ileum	
		Monkey†	Guinea pig
Ca.	0	> 1:1,000	N.D.‡
Chr.	0	1:1,000	1:10
Hun.	0	1:1,000	1:10
Ron.	27	1:1,000	1:100
Fei.	81	1:5,000	1:100
Vel.	81	> 1:1,000	1:500
Rog.	243	1:5,000	1:500
Hoa.	243	1:1,000	1:100
Hus.	243	1:5,000	1:10

*Reciprocal of highest dilution of serum that produced positive passive transfer in monkey skin.

†Highest dilution of serum which sensitized ileum of monkey and guinea pig in vitro.

‡N.D., Not done.

Inhibition of sensitization

Sera from several nonatopic individuals which did not produce in vitro sensitization of either monkey or guinea pig ileum were used in preliminary experiments. The undiluted normal serum (1 ml.) was added to strips of ileum from both the monkey and guinea pig for one hour at 37° C. Then the serum, diluted 1:100, of an untreated ragweed-sensitive patient was added to these strips. A subsequent challenge with ragweed extract failed to produce the muscle contraction, whereas a strip not previously treated with normal serum did produce the expected reaction. Hence, the addition of normal human serum inhibited the sensitization of the ileum.

DISCUSSION

The data presented definitely indicate that the sera from untreated atopic individuals can passively sensitize the ileum of both the monkey and guinea pig in vitro. However, there are several differences between the reactions obtained

with these two species. Several sera which were able to sensitize the monkey ileum in high dilutions did not sensitize the guinea pig tissues. Some of these sera (Table III) had negative or very low hemagglutination titers. It was likewise observed that heating the reaginic sera at 56° C. for 2 hours almost completely destroyed their ability to sensitize the monkey tissue but had little effect on their potential for sensitizing the guinea pig ileum. The above data strongly support the hypothesis that there are at least two types of antibody present in the sera of untreated atopic individuals. Accordingly, we have already reported³ that there are at least two antibodies in the sera from untreated ragweed-sensitive patients, determined by radioimmuno-electrophoresis. It may be assumed that the heat-labile factor represents reaginic antibody and that the monkey ileum is capable of fixing this antibody. A second, heat-stable antibody attaches to guinea pig ileum. It may be identical to the principal antibody that has been noted in the sera of untreated ragweed-sensitive patients by both tanned-cell and B.D.B. hemagglutination. In addition, these heat-stable antibodies may be responsible for the small muscular contraction of the monkey ileum that persists with some human sera even after prolonged heating.

As would be anticipated, heating antiragweed rabbit sera had no effect on the *in vitro* sensitization of either monkey or guinea pig ileum. For the most part, we used human reaginic sera that had been stored for some time. We also performed experiments with fresh serum for comparison. There was no apparent difference in their ability to sensitize the muscle strips. Hence, the storage of the sera at 4° C. had no effect on their sensitizing ability.

The relative sensitivity to the *in vitro* sensitization of the monkey and guinea pig tissue must be considered. The guinea pig ileum, under identical experimental conditions, was found to be at least from 10 to 100 times less sensitive than that of the monkey. This factor is probably much greater as the amplifications used when testing the guinea pig ileum were 10 times that employed when the monkey tissue was used. Some of this apparent difference in sensitivity could be related to the amount of tissue since the mass of the monkey strip is several times greater than that of the guinea pig. Successful passive sensitization *in vitro* of the guinea pig ileum with untreated human atopic sera seems not to have been previously reported. We observed that the ink-writing polygraph isometric contraction method was more sensitive than the classical apparatus usually used for these experiments.

Our data indicate that these reactions are specific as evidenced by several types of controls, including sera from European patients never exposed to ragweed, the sera from atopic individuals sensitive to substances other than ragweed, and the use of several different testing antigens.

The technique of *in vitro* sensitization of monkey ileum is apparently much more sensitive in detecting antibodies in atopic sera than is the usual passive transfer test in either human or monkey skin. However, this assay has rather large variability in sensitivity among different strips of ileum. Hence the contraction responses to histamine must be used for each strip in order to quantitate the reactions. This problem could be minimized by performing quantitative tests with several strips of ileum. This biologic difference is not unusual. Stanworth¹⁷

has reported the large discrepancies found when the different recipients for the usual passive-transfer tests in man are employed.

Extracts of ragweed pollen and *Alternaria* had to be used in high dilution in order to avoid the nonspecific contraction observed with greater concentrations when they are added to unsensitized muscle strips. We observed that dialysis of these extracts against cold distilled water for 48 hours removed this property. The effects were probably due to some dialyzable substances of low molecular weight which have histamine-like activity and are normally present in crude extracts. Extracts of house dust, timothy grass, feathers, and caddis fly could be used in these experiments in rather concentrated form without producing nonspecific contractions of unsensitized smooth muscle. We did observe, however, that when excessive concentrations of these antigens were employed, there appeared to be an inhibitory effect on these reactions. A similar phenomenon has recently been reported by Liacopoulos and associates.¹⁸ Further investigations on this subject are presently under study.

The observations that whole normal human serum can inhibit the in vitro sensitization with atopic serum confirm the results of other investigators and open a wide vista for future study. It has been demonstrated that gamma-2 globulin can inhibit the in vitro sensitization of the guinea pig ileum when a precipitating system is used.¹³ Ishizaka¹⁹ has reported that gamma-1A globulin will inhibit passive transfer of reagins to human skin. At the present time experiments are in progress with various fractions of normal human serum to test the inhibition properties in both the guinea pig and monkey systems.

Other studies underway concern fractionation of the reaginic sera, observing which fractions will sensitize the monkey or guinea pig ileum strip. The antigenic properties of various purified fractions of ragweed pollen extracts are also under investigation with the use of this technique. Results of these projects will be presented in subsequent reports. It is tempting to speculate whether this new investigative tool for allergic and immunologic problems might perhaps some day become a diagnostic aid for the study of allergic patients.

SUMMARY AND CONCLUSIONS

1. In vitro sensitization of monkey and guinea pig ileum by sera from untreated atopic patients was performed regularly and reproducibly. Especially in the case of monkey ileum the reactions were specific and sensitive.

2. When atopic serum was heated at 56° C. for 2 hours it almost completely lost its ability to sensitize the monkey ileum, but heating did not greatly affect sensitization of the guinea pig ileum.

3. These techniques probably measure at least two types of antibodies present in the sera of untreated atopic individuals.

4. Normal human serum can prevent in vitro sensitization by the atopic serum of either monkey or guinea pig ileum.

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