

In vitro lymphocyte transformation in atopic patients: Induced by antigens

*Jean Pierre Girard, M.D., Noel R. Rose, M.D., Ph.D.,
Marie L. Kunz, M.D., Setsuo Kobayashi, M.D., and
Carl E. Arbesman, M.D., Buffalo, N. Y.*

The effect of addition of antigens to cultures of lymphocytes from atopic individuals was investigated. The number of large "blast cells" in cultures of patients sensitive to ragweed, Alternaria, and penicillin was greater than in control cultures from non-sensitive individuals, supporting the specific stimulatory effect of antigen. For each antigen, there is an optimal dose-response curve. Above this optimal concentration no further stimulatory effect is obtained. When two or more antigens to which a patient is sensitive were added to his lymphocytes, no cumulative stimulatory effect was obtained, as compared with the effect of only one antigen. A similar absence of summation could be seen when an antigen was mixed with phytohemagglutinin. Although the morphology of the blast cells obtained with antigenic stimulation was similar or identical to that obtained with phytohemagglutinin (PHA), the percentage of blast cells was strikingly higher with PHA, and the peak of response appeared after 3 days with PHA instead of 5 to 7 days as with antigens. When the percentage of blast cells obtained with ragweed and Alternaria was compared with other tests of hypersensitivity, such as, skin test and passive hemagglutination, a fairly good correlation was observed. On the other hand, penicillin-sensitive patients can exhibit a positive hemagglutination test and an increased percentage of blast cells with a negative skin test. When antigen was added to the washed lymphocytes of a patient sensitive to that particular antigen, the culture fluid contained antibodies demonstrable by the Schultz-Dale technique. The highest content was observed from the third to the fifth day with a significant decrease at the seventh and ninth days. Heating this culture fluid at 56° C. for 4 hours significantly decreased the content in antibodies. The addition of fluid from lymphocytes grown in tissue culture in the presence of antigen caused contraction of unsensitized monkey ileum strip. This conceivably could be due to the presence of antigen-antibody complexes.

From the Departments of Bacteriology and Immunology and of Medicine, State University of New York at Buffalo and the Allergy Research Laboratory of the Buffalo General Hospital.

Supported by United States Public Health Service Grants AI-01303 and CAO-5203 and by Training Grant 5 T1 AI-51, National Institutes of Health.

Presented in part at the Annual Meeting of the American Association of Immunologists, April, 1965, Atlantic City, N. J., and in part at the Annual Meeting of the American Academy of Allergy, February, 1966, New York, N. Y.

Received for publication June 3, 1966.

Phytohemagglutinin, an extract of the red kidney bean, *Phaseolus vulgaris*, was used many years ago by Landsteiner¹ as an agglutinating agent for red blood cells. In 1959, Hungerford and co-workers² discovered that this extract, when added to tissue culture, induced transformation of 70 to 90 per cent of human peripheral lymphocytes into immature pyroninophilic blast cells capable of undergoing mitosis. Without the addition of this substance, only 5 to 15 per cent of the lymphocytes underwent these morphological changes in culture.

This discovery stimulated search for other factors which might exert similar activity. The first known blastogenic agent, other than phytohemagglutinin (PHA), was purified tuberculin (PPD), which stimulated lymphocytes from tuberculin-sensitive patients.^{3, 4} Soon after this action of tuberculin was discovered, evidence was presented that polio vaccine, tetanus toxoid,⁵ diphtheria toxoid, pertussis vaccine, and penicillin⁶ also induced blastogenesis of lymphocytes from donors specifically sensitized through natural infection or prophylactic immunization.

There are only a few studies regarding lymphoblastogenesis in relation to immediate hypersensitivity in man. To our knowledge, Elves⁷ and Zeitz⁸ were the first to report a specific stimulatory effect of grass pollen extracts upon lymphocytes of allergic individuals. We employed this technique to study in detail the effect of specific antigen on the lymphocytes from patients with hay fever, asthma, and urticaria. Since it was recently suggested by Hulliger and Sorkin⁹ and Landy and colleagues¹⁰ that leukocytes are able to elaborate specific antibody in vitro, we also studied the tissue culture fluid for presence of antibody.

MATERIALS AND METHODS

White blood cells from 48 patients, allergic to one or more antigens, and from 12 nonatopic individuals, were obtained and grown in tissue culture according to the following technique, which represents a modification of that described by Bach and Hirschhorn.¹¹ Ten to 20 ml. of blood was drawn under sterile conditions from the antecubital vein, with the use of a glass syringe previously wet with heparin. The blood sample was then allowed to sediment at 37° C. for one hour. The plasma was removed and put in a 4 oz. soft glass prescription bottle for one hour at 37° C. to allow attachment of polymorphonuclear leukocytes to the glass wall. The supernatant, which contained 60 to 90 per cent mononuclear cells, primarily lymphocytes, was centrifuged, and the cells were washed 3 times with Eagle's (MEM) medium.* After the last washing, the cells were counted and a final suspension containing 7.5×10^5 mononuclear cells per milliliter was prepared in MEM "spinner" medium supplemented with 1 per cent glutamine and 20 per cent fetal calf serum. No antibiotics were added to the culture.

Screw-cap tubes were used for the cultures, each containing 4 ml. of the final lymphocyte suspension. For each patient studied, phytohemagglutinin P (PHA-P),† 0.05 ml. per 10 ml. of culture, was added in one tube. Other tubes were prepared with various antigens in optimal concentrations, using antigens to which the patient may or may not have been allergic. Additional tubes without

*Grand Island Biological Laboratory, Grand Island, N. Y.

†Difco Laboratories, Inc., Detroit, Mich.

additives were included as controls. Cultures were incubated at 37° C. and harvested at different times (generally after 3 days for PHA studies and after 5 days when the antigens were used as the stimulus). In order to determine the time-response curve, the cells of 6 patients were harvested every day for 9 days. At the time of harvest, the cells were fixed with a mixture of glacial acetic acid and absolute methanol (1:3), and slides were prepared. Acetic orcein, a nuclear stain, was used for staining the cells. The nuclei of nontransformed lymphocytes remained compact and dark, while the "blast" cells showed a reticulated appearance with prominent nucleoli in most cells.* Five hundred to 1,000 cells were counted on each slide and the percentage of "transformed" (i.e., enlarged mononuclear) cells and cells exhibiting mitosis were recorded.

The tissue culture fluids were used for the determination of antibodies. After centrifugation at high speed and dialysis for 48 hours against buffered saline and concentration to half volume by evaporation, the samples were sterilized through a membrane filter. Intradermal skin tests were performed with this material on nonatopic individuals with the idea of determining the presence of antigen-antibody complexes, which have been shown to produce wheal and flare reactions on human skin.¹² In an attempt to demonstrate skin-sensitizing antibodies in the tissue culture fluid, indirect skin tests, according to the technique of Prausnitz-Kustner, were carried out. The tanned-cell passive-hemagglutination test was carried out with 10 samples of tissue culture fluid by the technique previously described.¹³ The tissue culture material from 3 patients was concentrated tenfold by pervaporation in the cold and used for the radio-immuno-electrophoresis technique with I¹²⁵-labeled Pool C, a partially purified fraction of ragweed.¹⁴

We have previously reported¹⁵ that the monkey ileum can be passively sensitized in vitro by as little as a 1:10,000 dilution of reaginic sera. The addition of the specific antigen then produced a muscle contraction which can be evaluated in terms of histamine equivalents. The supernatant fluid of the lymphocytes grown in tissue culture (with and without the addition of the specific antigen) was collected after 5 days' growth from 12 ragweed- and 2 penicillin-sensitive patients, as well as from 5 nonallergic individuals. These fluids were tested for the presence of antibody by this technique. In order to determine the effect of heating on the ability to sensitize the monkey ileum, samples of tissue culture fluid were heated at 56° C. for 4 hours and thereafter used in varying dilutions for the passive sensitization of the monkey ileum.

These tissue culture fluids were also tested by adding them to a muscle bath containing a nonsensitized strip of monkey ileum. Appropriate controls, such as normal tissue culture fluid, or the antigen alone, were used to challenge the unsensitized gut.

RESULTS

The lymphocyte cultures of a total of 48 allergic patients were studied, of which 32 were sensitive to ragweed, 6 to *Alternaria*, and 10 to penicillin. Six of these patients showed hypersensitivity to two of these antigens. The patients

*Although the origin and nature of these large mononuclear cells is uncertain, they are referred to throughout this paper as "blast" or "transformed" cells.

Table 1. Antigen-induced blast transformation of human peripheral white blood cells: Ragweed and *Alternaria* sensitive patients

Pa- tient	Skin reactivity*		Injec- tion therapy†	Blasts‡ after 5 days' culture (%)					Phyto- hemagglu- tinin P
	Rag- weed	Alter- naria		No antigen	Ragweed	Alternaria	Penicilloyl polylysine		
Cu	4+		+	12	62			83	
Cz	4+		—	19	70			89	
Ba	2+		—	16	39		22		
Ro	2+	2+	+	18	62	19	28		
Fa	4+	3+	+	19	53	59	38		
Li	4+	2+	—	17	33	29			
Ta	2+		—	24	43	22	22		
Ku	2+		+	31	53				
Jo	4+		—	6	29	20			
Mc	2+	3+	—	10	17	38	7		
Ba	2+		—	13	49	18		82	
Be	2+	2+	+	16	49	28			
Ha	4+		+	14	57	31			
He	3+		+	21	52	12			
Sc	4+		—	11	25				
Cl	4+	2+	—	13	28	36			
Wi	4+		+	17	41	36		74	
Mi	4+		+	36	68				
Fi	4+		+	8	56				
Gr	4+		—	15	15				
Do	4+		+	14	82				
Ot	4+		+	12	31				
Pa	4+		+	8	58				
Jo	4+		+	8	70				
Cu	4+		+	17	71				
Hi	4+		—	9	22			91	
Sm	4+		+	20	32			88	

*Based on the concentration of antigen to which the patient had a positive skin test. A reaction to a 1:1,000 dilution (30 PNU) or greater dilution of a 5 per cent extract of whole ragweed pollen is designated 4+ sensitivity; 1:100 dilution, 3+; 1:10 dilution, 2+ (3,000 PNU).

†The + indicates previous specific hyposensitization therapy, and the — indicates no previous specific hyposensitization therapy.

‡The results recorded represent the percentage of "blast" type cells observed in a total of 500 to 1,000 lymphoid cells counted. The italicized numbers indicate those instances in which the percentage of blasts upon stimulation was almost double the control level. This order of response is regarded as generally outside the limits of error inherent in the procedure. (Ragweed: Skin testing was performed with extracts of whole short ragweed pollen. In almost all cases, the ragweed antigen added to the lymphocytes in culture was Pool C, a partially purified fraction of short ragweed pollen, in a dose ranging from 0.5 mcg. to 20 mcg. per milliliter of culture medium. *Alternaria*: 0.1 ml. of aqueous extract containing 0.5 per cent of protein was added to the lymphocytes in culture. Dilutions of a 3 per cent extract were recorded as with ragweed for intradermal skin tests, namely, a skin reaction with a 1:1,000 dilution was recorded as 4+. Penicilloyl polylysine: .01 ml. of a 12×10^{-5} M added to 4 ml. of culture. Phytohemagglutinin P: Difco Laboratories, Inc., Control No. 479934.)

were carefully selected clinically and those with positive skin tests without a corresponding clinical picture were not included in this study. Lymphocyte cultures of 12 nonatopic individuals were used as controls.

In the presence of antigen, a portion of the cells showed morphological changes. The nucleus was enlarged and showed a reticular appearance with nucleoli prominent in most cells. These cells are strongly basophilic and some of them contained numerous cytoplasmic granules. The appearance of these cells

Table I—*Cont'd*

Pa- tient	Skin reactivity*		Injec- tion therapy†	Blasts‡ after 5 days' culture (%)				
	Rag- weed	Alter- naria		No antigen	Ragweed	Alternaria	Penicilloyl polylysine	Phyto- hemagglu- tinin P
Fr	4+		+	9	10			91
We	4+		+	13	13			90
Eu	0		—	21	29			
Ke	0	4+	—	14		26		73
Se	0		—	22	28	34		
Mi	0		—	21	21			

is suggestive of lymphoblasts, and, consequently, the term "blast cells" is used in this paper to characterize them. However, under conditions of the experiments, we were not able to distinguish lymphoblasts from other large, mono-nuclear cells such as macrophages. Without addition of antigen, only 15 per cent of the cells showed this type of morphological appearance.

The percentage of blast cells obtained in all three antigenic systems studied in detail is illustrated in Tables I and II and Fig. 1. Data on patients allergic to ragweed and/or *Alternaria* are listed in Table I. Studies of patients with recent various types of reactions following the administration of penicillin are presented in Table II. The fact that only lymphocytes of atopic patients respond to these changes upon the addition of the specific allergen is diagrammatically illustrated in Fig. 1. As controls, cells from nonatopic individuals, or of atopic patients not sensitive to the particular antigen, were treated with antigen. Generally, when the number of blast cells in cultures of control and sensitive patients are compared with and without addition of antigen, only the cells from sensitive individuals were consistently stimulated. There was approximately double the percentage of transformed lymphocytes in tissue cultures in the presence of antigen in 26 of 32 ragweed- and 7 of 10 penicillin-sensitive patients. Large variations and occasional overlapping can be seen (especially with *Alternaria*, Fig. 1) which could represent the result of a nonspecific stimulatory effect of the antigen used. There was no obvious reduction of blast response to previous injection therapy (Table I). Patients with various types of penicillin reactions responded more or less equally to either penicillin G or penicilloyl polylysine.

For each antigen, there seems to be a range of concentrations producing a maximal blastic transformation. When higher concentrations were used, a plateau was observed until toxic effects appeared. In Fig. 2 is represented the average percentage of transformed cells from 4 ragweed-sensitive patients, harvested after 5 days of culture. Pool C in a concentration of 0.5 μ g per milliliter produced maximal stimulation. Even 10 times this amount (5.0 μ g per milliliter) did not enhance this effect. The following concentrations of antigens were usually added to 4 ml. of culture media: penicillin, 0.1 ml. containing 1,000 units per milliliter; penicilloyl polylysine (PPL), 0.01 ml. of 12×10^{-5} M solution; and *Alternaria*, 0.1 ml. of aqueous extract containing 0.5 per cent of protein.

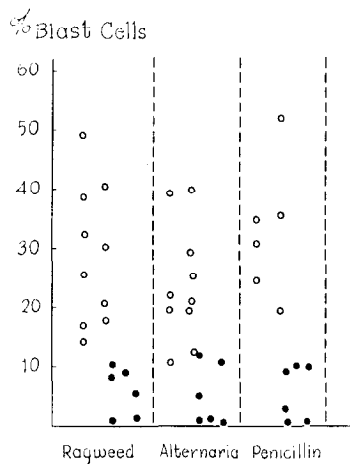


Fig. 1

Percentage of transformed lymphocytes after 5 days of culture, when *ragweed*, *Alternaria*, and *penicillin* antigens are added. Open circles indicate patients sensitive to that particular antigen. Filled circles indicate nonatopic patients or atopic patients not sensitive to that particular antigen

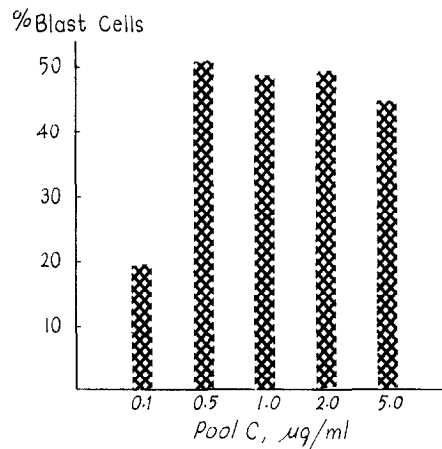


Fig. 2

Effect of various concentrations of *Pool C* (a partially purified fraction of ragweed) on the production of transformed lymphocytes. Average percentage of transformed cells from 4 ragweed sensitive patients harvested after 5 days of culture.

The effect of several antigens upon the same culture was then investigated. Mixtures of ragweed with penicillin, and *Alternaria* with Hormodendrum, were added to the cultures of patients allergic to both of these antigens. Mixtures of *Alternaria* with PHA were also tested. As demonstrated in Table III, no summation of their separate effects was obtained with either of the two antigen systems, or with the mixture of PHA with an antigen. In a further study, lymphocytes from a patient sensitive to several antigens were cultured in the presence of one of these antigens alone, or a mixture of them, as well as with PHA and with PPD alone or mixed with the antigens. No further stimulation

Table II. *Antigen-induced blast transformation of human peripheral white blood cells: Penicillin sensitive patients*

Patient	Sensitivity*	Skin reactivity		Blasts† after 5 days' culture (%)			
		Benzyl-penicillin G	Penicilloyl polylysine	No antigen	Benzyl-penicillin G‡	Penicilloyl polylysine	Phytohemagglutinin P
Jo	Skin rash	Neg.	Neg.	19	38		
Bi	Fever plus eosinophilia	Neg.	Pos.	8	28	41	
Sm	Anaphylactic reaction	ND§	ND	10	45	38	
Bl	Anaphylactic reaction	Neg.	Neg.	7	56	72	59
Ci	Skin rash	Pos.	Pos.	10	25	27	
Du	Skin rash	Neg.	Neg.	28	54		
Ne	Skin rash (receiving steroids)	ND	ND	30	29		90
Yo	Skin rash	Pos.	Pos.	16	27		
St	Skin rash	Neg.	Neg.	16	30		
Ce	Skin rash	ND	ND	18	19		91
Da	Nonsensitive			29	24		
Na	Nonsensitive			26	31		
Be	Nonsensitive			26	29		
Ha	Nonsensitive			12	8		
Da	Nonsensitive			26	20		
Mc	Nonsensitive			6	6		

*Type of allergic reaction to penicillin.

†See footnote to Table I.

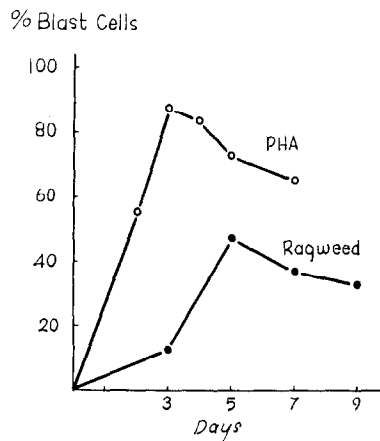
‡The usual concentration in culture was 250 units per milliliter of culture medium.

§ND, not done.

of the cells occurred with the addition of the mixture of several antigens, or by the addition of the antigen plus PHA.

The morphology of the blast cells induced by antigen is similar to that observed under the influence of PHA. There are however, some striking differences between the two systems. Fig. 3 illustrates two of these differences. First, the number of transformed cells is always much lower when the antigens are added than with PHA. Another difference resides in the time-response relationship. (With PHA, a peak number of blast cells is seen after 3 days in contrast to 5 to 7 days with the ragweed, *Alternaria*, or penicillin.) In the same culture the percentage of transformed cells remained practically unchanged from the fifth to the seventh day, but after this lapse of time there was a definite decrease in the number of cells, and degenerative processes could be seen.

Another way of testing the specificity of the cell transformation described above is to determine whether these morphological changes were related to other criteria of hypersensitivity. Direct skin tests and for the ragweed system the passive hemagglutination tests were compared with the number of transformed cells. There was some correlation between these three different tests, especially between the degree of skin hypersensitivity and the number of transformed lymphocytes. The blast formation in patients who developed strong positive skin reactions to a high dilution of antigen usually was much greater than that in patients with lesser skin reactivity.

**Fig. 3**

Time-response curve of "blast" formation following the addition of phytohemagglutinin (PHA) and Pool C.

Table III. The percentage of blast cells following the addition of 2 antigens to which the patient is sensitive, or antigen plus PHA

Patient	Antigens	Percentage of blast cells
Ja	Control	7
	Ragweed	38
	Penicillin	44
	Ragweed plus penicillin	41
Mo	Control	8
	Alternaria	22
	Hormodendrum	27
	Alternaria plus Hormodendrum	29
Gd	Control	11
	Alternaria	26
	Phytohemagglutinin	81
	Alternaria plus PHA	88
Wo	Control	18
	Alternaria	27
	Ragweed	32
	Horse dander	29
	Peanut	33
	All 4 antigens	29
	PHA	58
	All 4 antigens plus PHA	54
	Tuberculin (PPD)	38
	Ragweed plus PPD	33
	Ragweed plus PHA	55

In studies with the penicillin-sensitive patients, the correlation between skin tests and the percentage of transformed cells is not as striking. Some patients with penicillin hypersensitivity lacked skin sensitizing antibody, having only precipitating antibody as demonstrated by the passive-hemagglutination technique. Two of 5 patients receiving large doses of penicillin, but without a clini-

Table IV. *Blind experiment to assess antigen-induced blast transformation of peripheral lymphocytes from ragweed sensitive patients*

Specimen number	Patient	Blast cells after 5 days' culture (%) (Pool C)						Sensitivity
		No antigen	0.1 mcg./ml.	1.0 mcg./ml.	4.0 mcg./ml.	8.0 mcg./ml.	20.0 mcg./ml.	
1	Pa	8		<i>58*</i>				+
2	Jo	8		<i>70</i>				+
3	Cu	17		<i>71</i>				+
4	Do	5		8	<i>12</i>	<i>21</i>		-
5	Fi	8	<i>56</i>			<i>27</i>	<i>48</i>	+
6	Hu	12		8		7		-
7	Dom	15		<i>41</i>	<i>82</i>	<i>72</i>		+
8	Do	20	11		18	10	20	-
9	Ot	12		31				+

*Italicized figures denote at least twice the number of transformed cells in tissue culture with antigen than controls without antigen.

cal picture of hypersensitivity, developed a slightly higher percentage of blast cells than most normal control subjects.

In order to test the validity of this technique, further experiments were performed in a "blind" fashion, whereby cells were collected from 9 individuals (6 patients allergic to ragweed and 3 nonatopic patients) by one investigator. These samples were randomized and coded, and a second investigator tested these cells in culture by the addition of various concentrations of ragweed Pool C, as described above. The results of this study are illustrated in Table IV. All 6 of the ragweed-sensitive samples showed markedly elevated numbers of blast cells, whereas 2 of the normal samples showed no elevation and one showed slight response. It also will be noted, again, that increasing the amount of antigen added to the tissue culture above the optimal value did not increase the per cent of transformed cells.

The tissue culture fluids of lymphocytes from 7 patients sensitive to ragweed were studied for the presence of antibodies. All tests done with the passive-hemagglutination technique were negative. Direct skin tests performed for the determination of antigen-antibody complexes and passive transfer tests for the presence of antibody could not be interpreted because of the presence in all samples of a skin-irritating substance, which could be only partially removed by prolonged dialysis. Radioimmuno-electrophoresis was performed with the use of labeled Pool C with 3 samples of tissue culture fluids. Binding to proteins belonging to the group of alpha-globulin was observed, but no immunoglobulins could be detected.

The presence of antibodies in the tissue culture fluids of 12 ragweed- and 2 penicillin-sensitive patients was tested by the monkey ileum Schultz-Dale technique. The fluid was removed at different time intervals during lymphocyte culture, ranging from 4 hours to 9 days. Culture fluids, taken from antigen-stimulated lymphocyte cultures, sensitized strips of monkey ileum in vitro; upon subsequent addition of antigen, muscle contraction occurred. This contraction was

presumed to be due to the antibody present in the medium. One of these experiments is illustrated in Fig. 4, in which the histamine equivalents of the different samples are compared to that obtained with a 1:50 dilution of the patient's own serum. Tissue culture fluid from lymphocytes grown without addition of antigen was considered as a control.

The fluid obtained from lymphocytes grown in the presence of the specific antigen (Pool C) was able to passively sensitize the monkey ileum, as evidenced by a muscle contraction after the addition of the specific antigen. The supernatant fluid of cultures grown in the presence of an unrelated antigen did not sensitize the monkey gut to respond to challenge either by the same or unrelated antigens (Fig. 5).

Similar results were obtained in two other experiments which showed an increase in content of sensitizing antibody from the third to the fifth day, followed by a slight decrease by the seventh and ninth days. The amount of antibody detected with this technique was low. Usually, dilutions of the tissue culture fluid greater than 1:50 were not able to sensitize the monkey ileum. When the ileum was sensitized with tissue culture fluid in which only PHA was initially added, no muscle contraction occurred after challenge with the antigen.

Heating the culture fluid before using this material for the Schultz-Dale experiment produced a significant decrease in its ability to sensitize the monkey ileum. From 66 to 90 per cent of sensitizing activity is lost after heating the tissue culture fluid for 4 hours at 56° C. (Table V, Fig. 6).

To determine the validity of these results a blind test to demonstrate the presence of antibody in tissue culture fluids was performed. Lymphocytes from 4 ragweed-sensitive and 2 nonatopic individuals were collected and cultured with and without Pool C. The tissue culture fluids were separated after 5 days'

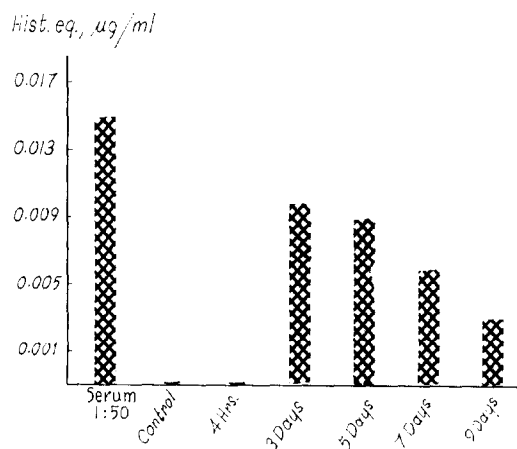


Fig. 4

Anaphylactic contraction in terms of histamine equivalents, obtained following passive sensitization of the monkey ileum by tissue culture fluid and subsequent challenge with the antigen. The result of a 1:50 dilution of the serum of a patient sensitive to ragweed is given and compared with that obtained with the culture fluid of his lymphocytes, in a 1:20 dilution, harvested at various periods of time from 1 to 9 days.

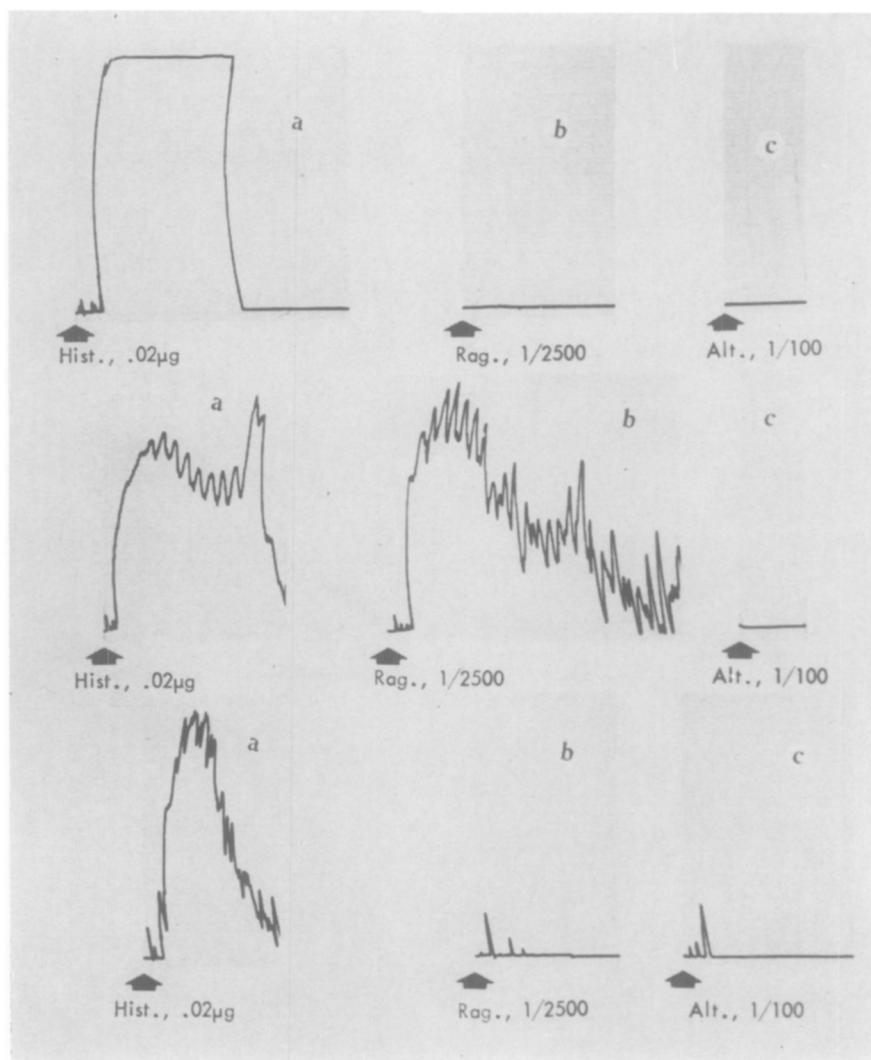
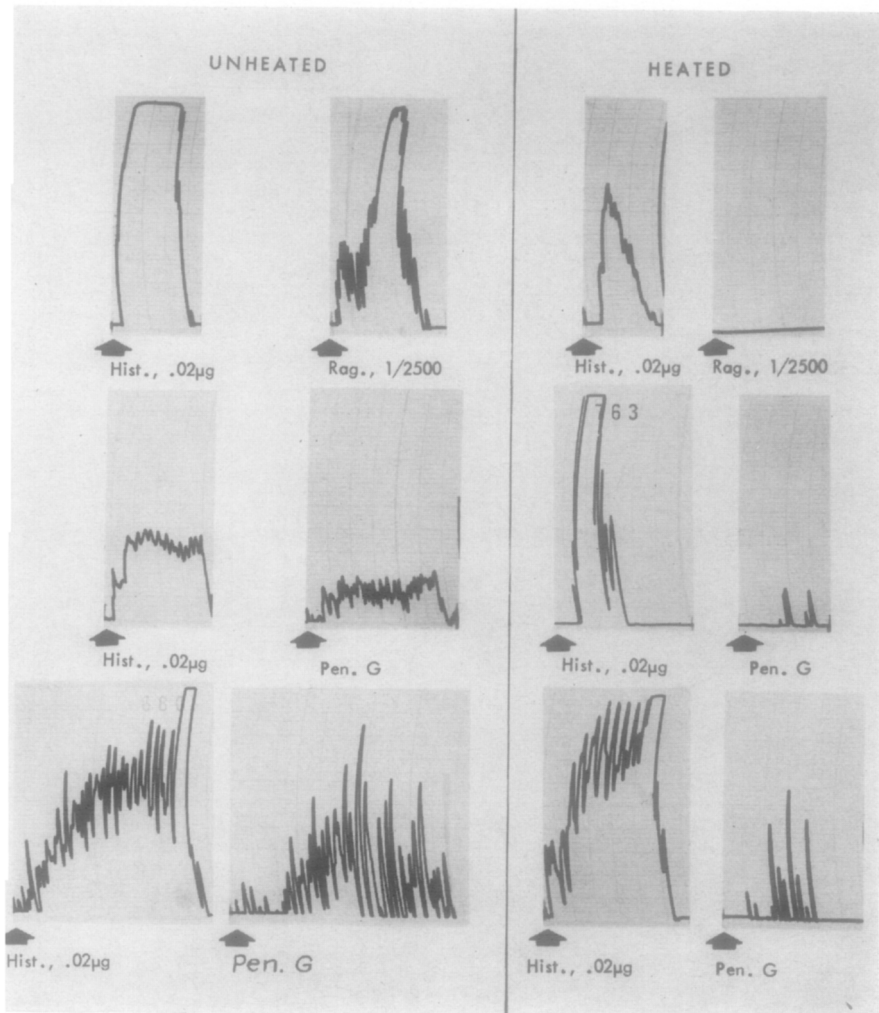


Fig. 5

To demonstrate the specificity of the tissue culture fluid to sensitize monkey ileum: Upper row of tracings show responses of monkey ileum sensitized with tissue culture fluid obtained from lymphocytes (grown without addition of Pool C) of ragweed-sensitive patient. *a*, Response to histamine; *b*, ragweed; and *c*, Alternaria. The middle row of tracings reveal the muscular response to the monkey ileum after being sensitized with the tissue culture fluid obtained from lymphocytes (grown in the presence of Pool C) of a ragweed-sensitive patient. *a*, Response to histamine; *b*, response to ragweed; *c*, response to Alternaria. The lower tracings illustrate the response to histamine, ragweed, and Alternaria after tissue had been sensitized with tissue culture fluid of lymphocytes (grown in the presence of Alternaria) from a ragweed-sensitive patient. *a*, Response to histamine; *b*, response to ragweed; *c*, response to Alternaria.

**Fig. 6**

The effect of heat (56° C. for 4 hours) on the ability of lymphocytes (grown in presence of specific antigen) of allergic patients to passively sensitize the monkey ileum. The upper tracings show response of ileum sensitized with *unheated* and *heated* tissue culture fluid from a ragweed-sensitive patient's lymphocyte culture. Although the histamine response is slightly diminished after heating, there is no response with the specific antigen (ragweed). The middle row of tracings shows responses from ileum sensitized with tissue culture fluid of penicillin-sensitive patient. Although the histamine response is not affected by heat, there is no response when penicillin is added. The lower row illustrates responses for another penicillin-sensitive patient's lymphocyte culture. A good response by the addition of penicillin to the strip sensitized by the *unheated* tissue culture fluid, but a markedly diminished response when the ileum was sensitized with tissue culture fluid heated at 56° C. for 4 hours.

Table V. *Schultz-Dale experiment with heated and unheated tissue culture fluid of lymphocytes from patients allergic to ragweed. The anaphylactic response obtained after challenge with the antigen is given in terms of histamine equivalents*

Patient	Histamine equivalents (ng./ml.)		
	Serum 1:50	Tissue culture fluid 1:20 (5 days)	
		Unheated	Heated at 56° C. for 4 hours
Sn	11	5	0.5
Ha	15	8	1.0
Hen	13	4	1.0
Sch	9	6	2.0
Ba	7	3	1.0

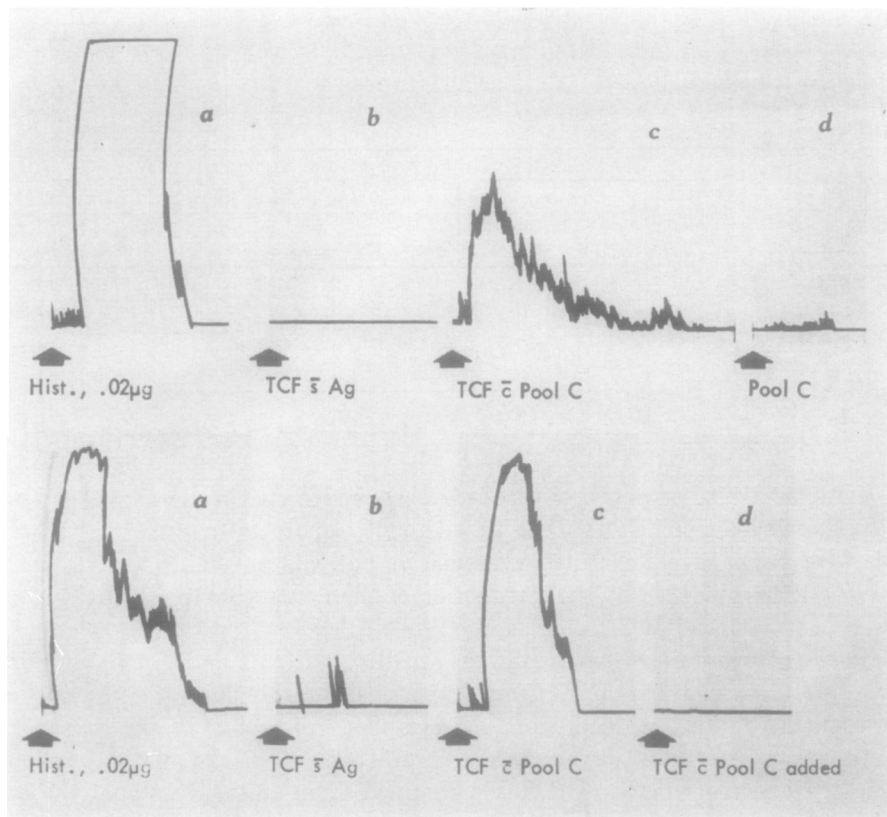
of growth, as described above, and coded. These coded specimens were then tested by the monkey ileum Schultz-Dale procedure by another investigator. Only the fluids with Pool C from the ragweed-sensitive patients were able to sensitize the monkey gut as evidenced by the contraction of the ileum after the addition of the ragweed extract.

Tissue culture fluids from 7 ragweed-sensitive patients and 2 nonatopic individuals were studied for their effects on unsensitized monkey gut. Six of the 7 tissue culture fluids obtained from the lymphocytes grown in the presence of the specific antigen for 5 days caused a significant contraction of the previously unsensitized monkey ileum. The fluids obtained from cultures without the presence of antigens did not produce this contraction, nor did the fluid from lymphocytes of the 2 nonatopic patients which had been cultured in the presence of antigen (Fig. 7).

DISCUSSION

In this study at least part of the white blood cell population of atopic patients recognized specific antigens such as ragweed and *Alternaria* and responded by characteristic morphological changes. The effects were apparently not attributable to previous injection therapy. Cell cultures from patients who experienced either immediate (anaphylactic) or late (serum-sickness or urticaria) reactions to penicillin demonstrated similar changes when either penicillin G or penicilloyl polylysine were added to the lymphocyte culture fluid. Cultures prepared from individuals who had received large dosages of penicillin without any allergic reaction showed no blastogenic response. These morphological changes are similar to those observed under the influence of PHA. However, the transformation pattern differed in many ways. PHA, which can be considered as a nonspecific stimulatory agent of the lymphocytes,¹⁷ was able to induce the metamorphosis of a higher percentage of cells and in a shorter time than any of the antigens tested in our experiments. In this respect, our results confirm previous observations.^{5, 6}

The addition to the culture of two or more antigens to which the patients are

**Fig. 7**

The effect of tissue culture fluid obtained from lymphocytes grown with and without the presence of antigen on a normal unsensitized monkey ileum strip. Upper row: *a*, histamine response; *b*, lack of contraction upon addition of tissue culture fluid obtained from lymphocytes grown without the presence of antigen; *c*, definite muscle contraction when tissue culture fluid was obtained from lymphocytes grown with specific antigen (Pool C); *d*, effect of antigen alone (same concentration as in tissue culture fluid). Lower row: *a*, histamine response; *b*, lack of contraction upon addition of tissue culture fluid obtained from lymphocytes grown without the presence of antigen; *c*, definite muscle contraction when tissue culture fluid was obtained from lymphocytes grown with specific antigen (Pool C); *d*, no response when antigen (Pool C in same concentration) was added to normal tissue culture fluid.

sensitive, or antigen plus PHA, did not increase the percentage of transformed cells, as compared with the effect of one antigen alone. This result suggests that only a limited proportion of the total lymphocyte population is involved in this process of blastogenesis and that the same cells are able to respond to several antigens. Similar results have been presented by Cowling and Quagliano¹⁸ who actually observed a decreased stimulatory effect when bacterial antigens were added to the culture, simultaneously with tuberculin, without being able to give an explanation to this peculiar phenomenon. These results support previous experiments demonstrating that the population of peripheral lymphocytes may be heterogenous in its function, stage of maturation, or immunological specificity.¹⁹

Especially surprising is the observation that blastogenesis induced in vitro

by either delayed or immediate hypersensitivity is similar as far as the morphology and the extent of transformation is concerned. Perhaps the same cells are able to respond to both types of immunological reactions and the basic cellular mechanism may be quite similar. Of course these assumptions need further documentation.

The origin and nature of these blast cells is still open to question. While they resemble lymphoblasts, the possibility that some of them represent macrophages or other mononuclear cells cannot be excluded. However, it must be emphasized that the proportion of these cells is not increased in cultures of blood cells from nonatopic individuals or in cultures with antigens to which atopic patients are not sensitive.

It was recently reported by Landy and co-workers¹⁰ that peripheral lymphocytes show the property of synthesizing antibodies in vitro. In our experiments, we were able to demonstrate in the fluid of lymphocyte cultures an antibody having the property of sensitizing passively the monkey ileum. This technique has previously been demonstrated to be an extremely sensitive test for antibody.¹⁵ Experiments with heated culture fluid showed that the antibody was heat labile, which is a common characteristic of the skin-sensitizing antibodies. Other techniques were employed, such as, passive hemagglutination, passive transfer, and radioimmuno-electrophoresis. All these experiments were negative. These negative findings are probably due to the small amounts of free antibody found in the tissue culture fluid.

In studies to determine the origin of these antibodies, the cells were carefully washed prior to being suspended in the culture medium, decreasing but not entirely ruling out the possibility of carrying over antibody from the patient's plasma. This possibility can be further excluded by the fact that very low titers, if any, were found in the culture fluid removed during the first day of culture. The time-response curve shows a peak at the third to fifth day and then a slow decrease in antibody content. The shape of this curve roughly resembled the time-response curve as obtained with the percentage of transformed cells under the influence of antigen. This peak with both the percentage of blast cells and the amount of antibody released suggests a relationship between the two phenomena. This timing is also in good accord with the general sequence of a secondary immunologic response. Our technique did not allow us to determine definitely if these antibodies are preformed in vivo, adhering to the cell wall, or contained in the cytoplasm and then subsequently released into the medium during the cellular metamorphosis, or, as a last possibility, if there was active antibody synthesis. It must be pointed out that, although the addition of PHA produces a large percentage of blast cells from lymphocyte cultures of patients allergic to ragweed or penicillin, no antibody was detectable in the culture fluid by the monkey ileum technique. Previously, other workers^{6, 17} were able to demonstrate active gamma-globulin synthesis during this cellular metamorphosis. Therefore, it is tempting to assume that the large cells observed are indeed antibody-forming cells. Richardson and Dutton²⁰ showed that in vitro antibody-forming cells appear only after many cells can be shown to be proliferating in response to antigen, suggesting that the blast cells are directly involved in synthesizing antibodies.

In the experiments described above, sensitization of the monkey ileum strip was accomplished by bathing the strips in the cell culture fluid in a test tube for 12 minutes at 37° C. This sensitized tissue was then suspended in the Schultz-Dale apparatus and antigen added. In some instances, the tissue culture fluid was added directly to an unsensitized strip suspended in the muscle bath. Contraction of the muscle occurred in six of the seven experiments. These contractions might be attributed to the presence of antigen-antibody complexes in the fluids, or other smooth muscle contractants released during growth of the cells in the presence of antigen. The fact that fluid heated at 56° C. for 4 hours did not produce contractions suggests that antigen-antibody complexes could well be responsible for the reaction.

Another possibility is the fact that a small number of polymorphonuclear cells are also present in the tissue culture fluid and the addition of the specific antigen could release histamine from the leukocytes into the fluid and cause a contraction when added to the unsensitized gut. The contraction could not be due to histamine release, as histamine is not inactivated when heated at 56° C. for 4 hours.

The technique of lymphocyte culture with its many variables is not yet completely standardized or understood and cannot be used routinely for diagnosis of hypersensitivity. However, the method is extremely sensitive, since only a very small amount of antigen is needed to stimulate the cells. In some cases, penicillin concentration as low as 50 units per milliliter induced transformation of sensitized cells. With the penicillin system, we learned that, if the patient is still receiving the antibiotic when the blood is drawn, several washings of the cells may not be enough to remove completely the antigen coating the cells. In fact, in the control samples of such lymphocyte cultures we sometimes observed a percentage of transformed cells higher than expected. These observations illustrate the high sensitivity of this technique but also emphasize the problem of unknown stimulatory factors which may be present in the patient's serum.

Finally, concerning the practical use of this technique, a few other limiting factors must be considered. According to our experience, some chemicals such as quinine or sulfonamides exhibit a cytotoxic effect on lymphocytes even when used in very low concentration, limiting their use as provocative antigens. Other drugs such as chloramphenicol²¹ may exhibit an inhibitory effect on the cell division. Finally, we have observed that several bean extracts, belonging to the family of legumes, have a nonspecific stimulatory effect on lymphocytes in culture, similar to that of phytohemagglutinin. This property must be remembered when using antigens of plant origin.

Some of the monkeys used in these studies were kindly supplied by Dr. Leon Schmidt of the National Primate Center, Davis, California.

REFERENCES

1. Landsteiner, K.: *The Specificity of Serological Reactions*, Springfield, Ill., 1936, Charles C Thomas, Publisher.
2. Hungerford, D. A., Donnell, A. J., Nowell, P. C., and Beck, S.: The Chromosome Constitution of a Human Phenotypic Intersex, *Am. J. Human Genet.* 2: 215, 1959.
3. Pearmain, G., Lycette, R. R., and Fitzgerald, P. H.: Tuberculin Induced Mitosis in Peripheral Blood Leucocytes, *Lancet* 1: 637, 1963.

4. Schreck, R.: Cell Transformation and Mitosis Produced In Vitro by Tuberculin Purified Derivate in Human Blood Cells, *Proc. Soc. Exper. Biol. & Med.* **113**: 191, 1963.
5. Elves, M. W., Roath, S., and Israels, M. E. G.: The Response of Lymphocytes to Antigen Challenge In Vitro, *Lancet* **1**: 806, 1963.
6. Hirschhorn, K., Bach, F., Kolodny, R. L., Firschein, I. L., and Hashem, N.: Immune Response and Mitosis of Human Peripheral Lymphocytes In Vitro, *Science* **143**: 1185, 1963.
7. Elves, M. W., Taylor, G., and Israels, M. E. G.: The In Vitro Production of Antibody by Lymphocytes, *Lancet* **1**: 1292, 1963.
8. Zeitz, S. J., McLure, D., and Van Arsdel, P. P.: Specific Response of Human Lymphocytes to Pollen Antigen in Tissue Culture, *J. ALLERGY* **36**: 197, 1965. Abst.
9. Hulliger, L., and Sorkin, E.: Synthesis of Antibody by Blood Leucocytes of the Rabbit, *Nature* **198**: 299, 1963.
10. Landy, M., Sanderson, R. P., Bernstein, T. M., and Jackson, A. L.: Antibody Production by Leucocytes in Peripheral Blood, *Nature* **204**: 1320, 1964.
11. Bach, F., and Hirschhorn, K.: Lymphocytes Interaction: A Potential In Vitro Histocompatibility Test, *Science* **143**: 813, 1964.
12. Ishizaka, K., Ishizaka, T., and Campbell, D. H.: The Biological Activity of Soluble Antigen-Antibody Complexes. II. Physical Properties of Soluble Complexes Having Skin Irritating Properties, *J. Exper. Med.* **109**: 127, 1959.
13. Arbesman, C. E., Rose, N. R., Kantor, S. Z., and Beede, R. B.: Immunologic Studies of Ragweed Sensitive Patients. I. Specificity and Sensitivity of Hemagglutination Reactions, *J. ALLERGY* **31**: 317, 1960.
14. Yagi, Y., Maier, P., Pressman, D., Arbesman, C. E., and Reisman, R. E.: The Presence of the Ragweed-Binding Antibodies in the B₂M-B₂A and Gamma Globulins of the Sensitive Individuals, *J. Immunol.* **91**: 83, 1963.
15. Arbesman, C. E., Girard, J. P., and Rose, N. R.: Demonstration of Human Reagins in the Monkey. II. In Vitro Passive Sensitization of Monkey Ileum with Sera of Untreated Atopic Patients, *J. ALLERGY* **35**: 535, 1964.
16. Goldfarb, A. R., and Callaghan, O. H.: Fractionation of the Antigen from Dwarf Ragweed Pollen, *J. Immunol.* **86**: 83, 1961.
17. Bach, F., and Hirschhorn, K.: The In Vitro Response of Peripheral Blood Lymphocytes. *Seminars in Hematology*, No. 2, January, 1965.
18. Cowling, D. C., and Quagliano, D.: Effect of Some Antigens on Leucocyte Cultures, *J. Path. and Bact.* **89**: 63, 1965.
19. Yoffey, J. M.: The Lymphocytes, *Ann. Rev. Med.* **15**: 125, 1964.
20. Richardson, M., and Dutton, R. W.: Antibody Synthesizing Cells: Appearance After Secondary Antigenic Stimulation In Vitro, *Science* **146**: 655, 1964.
21. Svehag, S. E.: Antibody Formation In Vitro by Separated Spleen Cells, Inhibition by Actinomycin and Chloramphenicol, *Science* **146**: 659, 1964.