

The in vitro blastogenic response of lymphocytes of ragweed-sensitive individuals

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Human peripheral leukocytes of normal and ragweed-allergic individuals were cultured with tritiated thymidine in vitro both in the presence and absence of the allergen and in the presence of autologous or homologous plasma. Cells of allergic individuals uniformly incorporated the labeled thymidine to a much greater degree than did cells of normal individuals. The optimal conditions for the incorporation of radioactive thymidine by allergic cells were 7 days in normal homologous plasma in the presence of 100 µg of allergen and with the isotope added 24 hours prior to termination of culture. Attempts to passively transfer blastogenic activity to cells of normal individuals were unsuccessful.

The diagnosis of the hypersensitivity state was until recently limited to the intracutaneous injection of the allergen into the patient and the subsequent appearance of the rapidly developing wheal and flare characteristic of immediate hypersensitivity (i.e., hay fever, asthma) or the slowly developing local induration of delayed hypersensitivity (i.e., tuberculin sensitivity). In the past decade, in vitro techniques have been introduced for the diagnosis of the hypersensitivity state inspired by the general desire to avoid manipulations of the patient. These techniques include the passive hemagglutination method (BDB¹ or tanned cell²), the release of histamine from passively sensitized leukocytes³ and the specific contraction of smooth muscle of primate origin.⁴ More recently, it has

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been demonstrated that lymphocytes of allergic individuals are capable of transforming to blast cells *in vitro* when incubated with the specific allergen.⁵⁻⁷ We have now extended our initial study and have attempted to evaluate this technique as an indicator of the allergic state.

MATERIALS AND METHODS

Peripheral white cells were obtained by bleeding untreated clinically allergic adults and normal healthy ones via the antecubital vein with a heparinized sterile syringe (100 U. of heparin per milliliter of blood). None of the allergic individuals had previously undergone desensitization treatment nor had they received any drugs or any other form of therapy for at least two to three months prior to the experiment. The blood was immediately introduced into sterile disposable plastic tubes (Falcon tubes) maintained at a 60 degree angle in a 37° C. incubator. At the appropriate time (45 to 60 minutes), the upper, white cell-rich plasma layer was decanted and a cell count performed by hemocytometer. The cells in the autologous plasma were then diluted with Medium 199 to contain 10⁶ cells per milliliter. Penicillin and streptomycin were added to give final concentrations of 100 U. per milliliter and 100 µg per milliliter, respectively. Four milliliters of this cell suspension was then introduced into individual plastic tubes, 0.25 ml. of the ragweed extract, made up to the specified concentration, was added, and the tubes were sealed tight and maintained in a vertical position at 37° C. All experiments were performed in triplicate. At the times indicated below, 0.1 ml. of a solution containing 20 µc of tritiated thymidine (specific activity, 5,000 µc per millimolar) and 10 µg of carrier thymidine was added to each tube.* At the end of culture, 100 µg of nonradioactive thymidine was added to each tube in order to decrease the extent of nonspecific adsorption of tritiated thymidine onto the precipitated cell mass, the tubes were centrifuged at 1,000 r.p.m. for 10 to 15 minutes, the supernatants discarded, and the cell button resuspended in 2 ml. of 5 per cent trichloroacetic acid. The tubes were centrifuged and washed once more in the same way with trichloroacetic acid. One-half milliliter of Hyamine† was then added to each tube, and they were permitted to digest for 24 hours at room temperature in the dark. The contents of the tubes were then transferred to scintillation counting vials with the use of two washes of ethyl alcohol (0.6 cc., total). The vials were then incubated at 70 to 75° C. for one hour and allowed to cool at room temperature, following which 15 ml. of scintillation solution (400 Gm. of naphthalene, 28 Gm. of PPO, and 1.2 Gm. of POPOP, made up to 3.8 L. with dioxane) were added to each. The vials were analyzed for their radioactive content in a Model 4000 Packard Scintillation Counter. The results are expressed as counts per minute. Only experiments in which the triplicate determinations did not vary by more than 5 per cent from each other were accepted for presentation. Less than 10 per cent

*Carrier thymidine was added along with the labeled thymidine since it has been demonstrated that the presence of only isotopically labeled thymidine exerts a toxic or inhibitory effect on the cell population, resulting in a diminished blastogenic response and incorporation of tritiated thymidine. The quantities of labeled and unlabeled thymidine used here have been found to give optimal results.

†Hydroxide of Hyamine 10-X, Packard Instruments.

of the experiments performed were rejected on this basis. These experiments were then repeated.

For radioautography, the tubes were centrifuged at 800 r.p.m. for 5 minutes, washed once with Medium 199, and smears made on gelatin-coated slides. The slides were air-dried, coated with Kodak NTB-2 Nuclear Track Liquid Emulsion, kept at 4° C. in light-tight slide boxes containing a desiccating agent (Drierite) for two to three days, and developed and stained with Jenner stain.

The ragweed preparation used* was the dialyzed residue of the water-soluble extract of ragweed pollen. It was lyophilized and stored at -10° C. until used.

In several experiments, the cells of the allergic donors were first centrifuged at 800 r.p.m. for 5 minutes, the autologous plasma discarded, and the cells diluted and cultured in medium containing homologous, nonallergic serum.

An attempt was also made to transfer blastogenic activity to normal lymphocytes by initial incubation with allergic serum. Three representative allergic sera were used. The donor cells were incubated with either 1 or 2 ml. of the allergic serum at 37° C. for 3 hours, washed with Medium 199, and cultured in medium fortified with homologous normal serum and the allergen (100 µg) for

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Table I. *Effects of variation of allergen concentration and time of incubation on the incorporation of radioactive thymidine by allergic lymphocytes in vitro*

Quantity of allergen added to culture tube* (µg)	Day tritiated thymidine added	Duration of culture (days)	Uptake of tritiated thymidine By cells of patient† (counts per minute)		
			B. V.	K. W.	J. W.
Nil	3	5	340	300	400
Nil	5	7	780	420	750
Nil	7,9	10	780	440	820
1	3	5	810	1,600	650
1	5	7	2,200	4,000	2,200
1	7,9	10	2,600	5,000	3,070
5	3	5	900	1,300	1,000
5	5	7	3,600	5,500	4,000
5	7,9	10	4,000	8,200	5,000
25	3	5	1,050	1,000	1,100
25	5	7	3,700	5,400	5,050
25	7,9	10	5,000	7,500	6,200
100	3	5	1,300	1,500	1,600
100	5	7	8,000	8,500	9,100
100	7,9	10	6,800	6,000	8,200
500	5	7	5,300	6,000	6,500
500	7,9	10	4,000	3,800	5,000
1,000	5	7	3,000	5,000	6,000
1,000	7,9	10	1,500	3,000	3,800

*Cells were incubated in medium containing autologous plasma.

†Each value represents the mean of triplicate determinations.

7 days. Tritiated thymidine was added at Day 6, the tubes were centrifuged and washed 24 hours later, and the cells were processed for radioactive thymidine incorporation in the manner described before.

RESULTS

The initial experiments involved the addition of varying quantities of the ragweed preparation to the tubes and removal of the tubes at different intervals of time. As can be seen from Table I, the optimal amount of allergen required to induce blastogenesis and mitosis with maximum incorporation of tritiated thymidine was 100 μ g and the optimal time of incubation 7 days. The incorporation of radioactive thymidine by the cells of allergic individuals, as compared to that taken up by cells of normal, nonallergic people, is presented in Table II. Interestingly enough, the specific uptake of isotope, represented by the ratio

Table II. *Incorporation of radioactive thymidine by cells of allergic and normal individuals incubated with allergen in vitro*

Cells of individual tested*	Uptake of tritiated thymidine by cells incubated in medium containing† (counts per minute)			
	Autologous plasma without allergen	Autologous plasma with allergen	Homologous plasma without allergen	Homologous plasma with allergen
Normal individuals				
A	2,600	2,750	2,400	4,600
B	2,300	4,500	1,350	4,550
C	2,600	9,200	2,700	8,650
D	6,300	6,200	3,700	9,100
E	1,200	1,000	1,000	1,050
F	4,400	4,200	1,350	3,300
G	2,000	12,000	2,600	10,000
H	1,550	1,900	1,200	2,000
I	2,200	1,900	1,700	2,450
J	375	575	400	700
K	950	3,300	1,150	3,100
L	88	105	120	250
M	2,000	1,700	1,300	2,150
N	775	875	985	1,050
O	700	3,200	2,600	7,500
P	850	5,100	1,950	6,500
Allergic patients				
A	210	275	560	1,500
B	4,200	33,000	2,300	22,000
C	1,000	9,000	1,600	21,000
D	360	2,000	530	10,000
E	1,800	4,200	1,200	8,800
F	1,300	8,000	1,350	11,000
G	1,100	2,600	1,300	3,700
H	210	600	225	2,100
I	1,130	5,500	2,000	8,800
J	650	6,800	1,310	8,900
K	1,080	1,650	950	5,200

*Duration of culture: 7 days. Tritiated thymidine added on Day 5.
†Each value represents the mean of triplicate determinations.

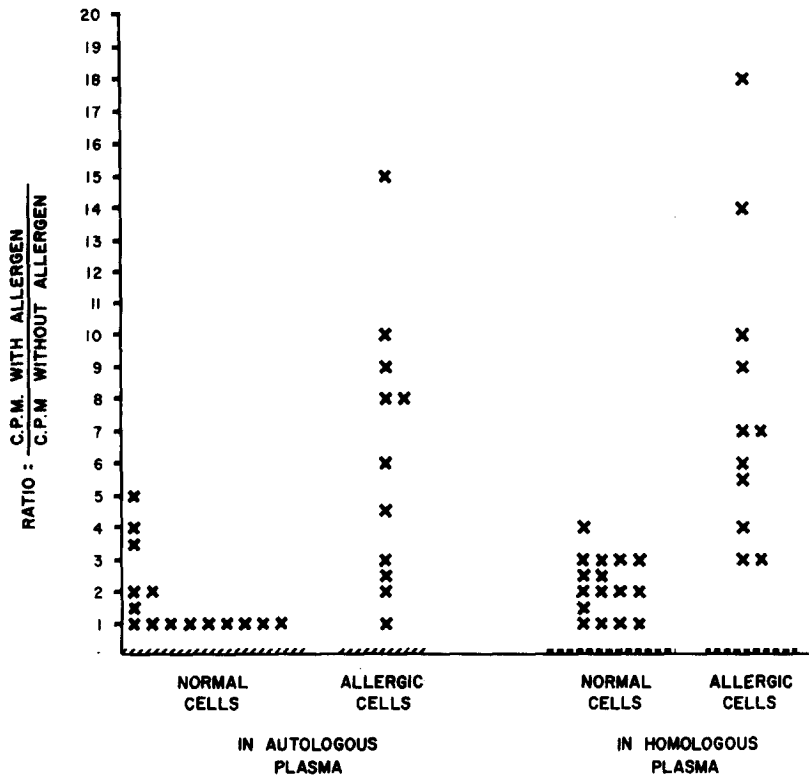


Fig. 1
Plot of specific uptake of tritiated thymidine by cells of allergic and normal individuals.

Table III. Effect of time of addition of tritiated thymidine on its incorporation by allergic lymphocytes incubated with allergen in vitro

Allergic cells incubated in medium containing:	Day allergen (100 γ) added	Day tritiated thymidine added	Day culture terminated	Incorporation of tritiated thymidine* (counts per minute)	
				Cells of A	Cells of B
Autologous plasma	0	5	6	16,000	27,000
	0	5	7	10,000	12,000
	0	6	7	22,000	45,000
	0	6¾	7	30,000	70,000
	Nil	5	7	800	1,300
	Nil	6	7	1,200	4,000
	Nil	6¾	7	1,800	2,000
Homologous normal plasma	0	5	6	18,000	23,000
	0	5	7	14,000	11,000
	0	6	7	28,000	42,000
	0	6¾	7	37,000	43,000
	Nil	5	7	1,000	1,000
	Nil	6	7	1,600	2,400
	Nil	6¾	7	2,200	1,600

*Each value represents the mean of triplicate determinations.

of incorporation of isotope in the presence of the allergen compared to that incorporated in its absence, was much greater when the "allergic" cells were washed and cultured in homologous, normal serum than if cultured in autologous plasma. This effect was not observed with cells of normal individuals. The data are presented graphically in Fig. 1; the results are plotted as the ratio of incorporation of tritiated thymidine in the presence of allergen to that incorporated in its absence (specific uptake). A standard t-test was performed on the data presented in Fig. 1 representing the experiments which involved the use of homologous serum rather than autologous plasma. It was determined that the probability of both sets of values, that is, cells of controls and cells of allergic individuals, having arisen from the same population was less than 0.001. These results are therefore considered to be highly significant. Although in the above experiments the tritiated thymidine was added on Day 5 of culture, the optimal time for its addition was later found to be 24 hours prior to the termination of culture. Two representative experiments are illustrated in Table III. In general, in spite of much greater incorporation of isotope into the culture, the specific uptake of the isotope as defined before was not increased significantly with the cells of the allergic individuals when compared to the uptake of thymidine when the isotope was added on Day 5 of culture.

The addition of the allergen to cells of allergic individuals (referred to as allergic cells in the tables) maintained in medium containing a subthreshold concentration of phytohemagglutinin (see Discussion) on Day 4 of culture did not result in any appreciable increase of thymidine incorporation by Day 11 of culture as compared to cells incubated with allergen during the usual 7 day culture period (Table I).

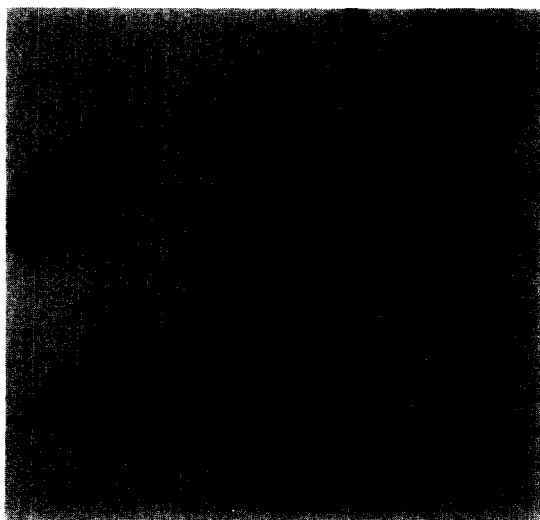


Fig. 2

Radioautograph of peripheral lymphocytes of allergic individual incubated for 7 days with the allergen. Tritiated thymidine added on Day 6.

Radioautographs of the cells at the termination of culture revealed a large number of transformed cells, all of which exhibited the same morphological and radioactive labeling characteristics as blast cells obtained after PHA stimulation (Fig. 2).

Attempts to passively transfer blastogenic activity *in vitro* were unsuccessful. As can be seen in Table IV, the incorporation of radioactive thymidine by normal cells incubated initially in the allergic serum for 3 hours, washed and then incubated for 7 days with the allergen was within the range of uptake by normal cells which had not been preincubated with allergic serum.

DISCUSSION

Various antigens have been shown to possess the capacity to stimulate peripheral blood lymphocytes obtained from immunized individuals to undergo blastogenesis and mitosis *in vitro*. In contradistinction to phytohemagglutinin (PHA), which induces the enlargement of 80 per cent or more of the small lymphocytes in 3 days of culture,^{8, 9} specific antigens stimulate the enlargement of 5 to 40 per cent of the cells after 5 to 10 days of incubation.^{5-13, 16-25} Lycette and Pearmain,¹⁰ in 1963, cultured lymphocytes obtained from five patients with

Table IV. *Passive transfer of blastogenic activity with sera of allergic individuals in vitro*

Initial incubation of normal cells for 3 hours at 37° C. in:	Incubation of cells for additional 7 days in homologous normal plasma		Uptake of tritiated thymidine added on Day 6† (counts per minute)
	With allergen* added on Day 0	With PHA added on Day 4	
Allergic serum A			
Diluted twofold	+	-	1,200
	-	-	2,200
Diluted fourfold	+	-	1,200
	-	-	2,500
Allergic serum B			
Diluted twofold	+	-	1,300
	-	-	1,100
Diluted fourfold	+	-	1,600
	-	-	1,600
Allergic serum C			
Diluted twofold	+	-	1,800
	-	-	1,000
Diluted fourfold	+	-	1,000
	-	-	500
Normal homologous serum			
Diluted twofold	+	-	1,000
	-	-	600
Diluted fourfold	+	-	1,200
	-	-	1,200
Autologous plasma	-	+	140,000
Homologous plasma	-	+	130,000

*One hundred micrograms per vial.

†Each value represents the mean of triplicate determinations.

severe grass hay fever. A polyvalent extract of grass pollens, which elicited strongly positive skin tests, was added to cultures. Blast cells and mitoses were observed after 4 to 6 days of incubation, although much fewer in number than in cultures of the same individual with PHA. In 1965, Wiener and Brasch¹¹ reported that the addition of grass pollen extracts to lymphocytes from sensitized individuals resulted in a significant increase in the number of mitoses. This mitogenic effect was greatly depressed when leukocytes of desensitized patients were used. Zeitz and colleagues,⁵ in 1966, observed blast cell transformation in cultures of peripheral lymphocytes obtained from timothy-sensitive individuals and incubated with timothy pollen extract. This response was not affected with hyposensitization treatment, thus failing to support the findings of Wiener and Brasch.¹¹ Girard and colleagues⁶ were also able to obtain blastogenesis of lymphocytes with ragweed pollen extract, *Alternaria*, and penicillin as antigens. On the other hand, Ricci and colleagues¹² were unable to induce blastogenesis in lymphocyte cultures from patients sensitive to Gramineae pollens when this antigen was added to the cultures. All of the above studies involved the morphological examination of smears of the cell suspensions representing only a small percentage (less than 0.1 per cent) of the cultured cells. The results were extrapolated to account for the behaviour of the entire culture and were expressed as per cent blast cells or mitoses. However, as has been pointed out by Caron,¹³ Elves,¹⁴ and Coulson and Chalmers,¹⁵ this technique of estimating the blastogenic response is highly subjective and subject to error. Furthermore, as stated by Girard and colleagues,⁶ it is sometimes very difficult to differentiate blast cells from other large mononuclear cells, such as macrophages, by the fifth day of culture on a purely morphological basis, and it is probable that various nonblast cells were designated as blast cells in the studies referred to above. The technique used in the present study, the incorporation of tritiated thymidine by the dividing cells and its determination in a scintillation counter, reflects the behaviour of the entire cell population and therefore circumvents the errors inherent in a purely morphological analysis. Using the technique of isotope incorporation, Mayron and Baram¹⁶ observed only a slight increase in the rate of nucleic acid synthesis when ragweed pollen extract was incubated with lymphocytes of allergic individuals in the presence of guanosine-C¹⁴.

Our data, obtained with the use of tritiated thymidine, do not agree with the results of Mayron and Baram.¹⁶ We have demonstrated that lymphocytes of ragweed-allergic individuals are capable of specifically undergoing transformation to blast cells when incubated with the allergen *in vitro*. Unlike the results of Girard and colleagues⁶ and Zeitz and colleagues,⁵ who observed optimal incubation periods of 5 and 10 days, respectively, maximum transformation in our cultures was observed to occur by Day 7. The blast cells observed in the cultures at Day 7 appeared morphologically identical to those observed after incubation with phytohemagglutinin. Examination of radioautographs revealed that almost all of the transformed blast cells had incorporated tritiated thymidine. Furthermore, no labeled small lymphocytes could be found. This observation suggests that the allergen cannot simply induce the small lymphocyte to enlarge and remain dormant *in vitro*. Once the blast stage is reached, replica-

tion of DNA takes place with ensuing mitotic division. In this respect, the cellular events observed here are very similar to those occurring in PHA cultures.

It is interesting to note that cells of allergic individuals transformed to a greater degree and incorporated more thymidine in the presence of the allergen when the medium contained normal homologous, rather than autologous, serum (Table II). By and large, the source of the serum in the culture medium did not appear to affect the uptake of tritiated thymidine by normal lymphocytes. The "inhibiting" effect on the blastogenic response by autologous allergic plasma may be attributed to antibodies in the medium (i.e., reagin and/or blocking antibody and/or hemagglutinating antibody). This inhibition may be due to neutralization of the allergen by the antibody in the medium, thus effectively preventing interaction between the lymphocyte and free allergen. However, it seems unlikely that the presence of antibody could account for the lesser response in the presence of autologous plasma, since the uptake of tritiated thymidine was not increased even when the allergen was used at a concentration of 1,000 μg per tube, which is about tenfold greater than the optimal blastogenic concentration (not shown in Table I). Although a number of the normal cell cultures incorporated tritiated thymidine to a considerable degree, relative to cells of allergic individuals, the basal mitotic activity of these cells, that is, the rate of transformation of these cells in the absence of the allergen, was also quite high. In order to take this latter factor into account, the results have been expressed as the "specific uptake" of tritiated thymidine, which is defined as the uptake of thymidine in the presence of the allergen divided by the incorporation of thymidine in the absence of the allergen. When these data are plotted as in Fig. 1, it is clear that the uptake of radioactive thymidine and transformation of allergic cells, as compared to control cultures, is most marked in medium containing homologous plasma. Only 3 of the 11 allergic cultures fell into the range of thymidine uptake of the control cultures. This effect was not as marked in medium containing autologous plasma. Statistical analysis of the data confirmed our interpretation as to the specificity of the thymidine uptake by the cells of allergic, as compared to the cells of normal, individuals in the presence of the allergen (probability < 0.001).

It has been demonstrated in this laboratory that PHA, used in a subthreshold concentration, is capable of maintaining lymphocytes in a viable and metabolically active state *in vitro* for periods of time in excess of 35 days. These cells do not survive in the absence of the PHA.³⁹ The subthreshold concentration of PHA was defined as the maximum concentration of PHA in the culture medium which will not induce blastogenesis and mitosis in excess of control cultures. Furthermore, we have also observed that cells maintained in subthreshold PHA appear to be more reactive to the subsequent addition of a suprathreshold (or mitogenic) dose of PHA, as compared to earlier cultures.³⁷ On the assumption that PHA may enhance the response of allergic lymphocytes to the allergen *in vitro*, allergic cells were maintained with subthreshold PHA only for the initial 4 days of culture. Allergen was added to the culture on Day 4, tritiated thymidine on Day 8 or 10, and the cells sampled on Day 9 or 11, respectively. The uptake of tri-

tiated thymidine by these cells, however, was not greater than that observed with the cultures during the normal 7 day incubation period.

We were unable to passively transfer blastogenic activity with allergic serum. Similar results using PPD and plasma of PPD-sensitive individuals have been reported by Mayron and Baram.¹⁶ It may be that our conditions were not optimal or that normal lymphocytes cannot be passively sensitized by allergic serum to undergo blastogenesis in the presence of the allergen. The source of the normal lymphocytes may be a critical factor. Levy and Osler³ stated that only one in every five nonallergic individuals provided suspensions of leukocytes suitable for passive sensitization with respect to histamine release in the presence of the allergen. It is possible that our failure to passively transfer blastogenic activity is related to the fact that we have so far tested the leukocytes of only two normal individuals.

The nature of the blastogenic response observed in the presence of allergen requires elucidation. Does it represent the capacity of the lymphocytes to form and secrete humoral antibody, or does it represent the delayed component of the immune response? Certainly enough positive results^{5-13, 16-25} regarding the efficacy of the blastogenic response of human lymphocytes to various allergens have appeared in the recent literature, with only several contrary ones,^{26, 27} that we cannot doubt the occurrence or specificity of this reaction. However, we cannot determine from these studies whether it is the cellular (delayed) or humoral response which is measured by the blastogenic phenomenon. Nevertheless, a definite correlation has been found between the capacity of the lymphocytes to undergo blastogenesis *in vitro* in the presence of various antigens and phytohemagglutinin (PHA), an extremely potent nonspecific blastogenic agent,^{8, 9} and the elicitation of a delayed skin reaction *in vivo*. Depressed *in vitro* responses to PHA and antigens have been demonstrated in patients with Hodgkin's disease,^{32, 33} sarcoidosis³⁴ chronic lymphatic leukemia,^{35, 36} and ataxia telangiectasia,^{37, 38} diseases which are characterized by impaired delayed hypersensitivity responses *in vivo*. Results of a more clear-cut nature have been obtained from experiments in animals. Mills²⁸ observed that lymphocytes from guinea pigs displaying delayed hypersensitivity to tuberculin underwent transformation on exposure to this antigen in cell culture whereas lymph node cells of animals which synthesized large quantities of humoral antibody to tuberculin failed to show transformation *in vitro*. Similar results were obtained by Oppenheim and colleagues.²⁹ These results suggest a direct relationship between delayed hypersensitivity *in vivo* and the capacity of the lymphoid cells to undergo specific transformation *in vitro*. However, the findings by Dutton and Eady³⁰ and Vischer and Stastny³¹, that lymphoid cells of previously immunized animals, reinjected to give a secondary antibody response, respond with thymidine uptake *in vitro* at about the same time that antibody can be detected in the circulation, suggest a direct relationship between blastogenesis and humoral antibody formation. Thus, although the animal experiments are individually more easily interpretable with respect to the nature of the *in vitro* blastogenic response, they in fact contradict each other. It is possible that the blast cell transformation reaction is measuring both cellular (or delayed) and humoral

immunity, since both of these activities are manifestations of lymphocyte activity.

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