References and Notes

1. E. R. Long, in *The Chemistry and Chemotherapy of Tuberculosis* (Williams and Wilkins, Baltimore, ed. 3, 1958), chap. 13.
2. S. D. Chaparas and H. Baer, *Am. Rev. Respirat. Diseases* **89**, 41 (1964).
3. W. Hartston and P. W. Muggleton, in *Trans. of the Health and Tuberculosis Conference*, held at University College, Ibadan, Nigeria, 26–31 March 1962.
4. Purified protein derivative prepared according to the method of Dr. Florence Seibert [see (*1*), p. 126].
5. M. Nomoto, Y. Narahashi, M. Murakami, *J. Biochem.* **48**, 593 (1960); ———, *ibid.*, p. 906.
6. A. R. Rich, *Pathogenesis of Tuberculosis* (Thomas, Springfield, Ill., ed. 2, 1951), p. 376.
7. H. Baer and S. D. Chaparas, *Am. Rev. Respirat. Diseases* **88**, 28 (1963).

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Phytohemagglutinin Elicitation of Specific Anamnestic Immune Response in vitro

Abstract. *Fragments of lymph nodes from rabbits hyperimmunized with human chorionic gonadotropin or bovine plasma albumin were cultivated in organ culture for 1 month. An anamnestic response was elicited in vitro by the sensitizing antigen. Phytohemagglutinin also produced such a response. Throughout cultivation the cultures showed excellent preservation of morphological integrity and of capacity to produce antibody.*

Nonspecific induction of a secondary response was taken as a matter of fact by early workers in the field of immunology (*1*). Later, it was concluded that only specific or cross-reacting antigen could elicit such a response (*2*). We used phytohemagglutinin P (PHA) (*3*) as a mitotic stimulant to elicit an anamnestic response in vitro; our results suggest that nonspecific elicitation may occur under these circumstances. This study attempted to determine whether physical presence of the antigen is necessary for elicitation and maintenance of the anamnestic response.

Adult female albino rabbits were used; each received intravenous injections totaling 2000 to 3000 international units (IU) of human chorionic gonadotropin (HCG) (*4*) or 25 mg of bovine plasma albumin (BSA) (*3*). Three to 6 months after the last injection, the peripheral lymph nodes were removed, pooled, and cut into fragments of 1 mm^3 for cultivation. All animals showed circulating antibody titers of 1 to 1280 to 1 to 20,480 at the time of death. An organ culture system requiring the use of glass beads and filter paper (*5*) was employed. The ratio of tissue wet weight to medium-volume was adjusted to 15 to 20 mg/ml. The culture medium was Eagle's minimum essential medium, supplemented with 20 percent normal rabbit serum and 100 IU of penicillin per millilter; it was renewed every 1 to 3 days. The cultures were treated in vitro with the antigen or PHA for 2 to 3 hours on day 0. Throughout the month of cultivation, antibody production of the fragments was followed by determining the antibody titer in the medium by means of the passive hemagglutination method (*6*).

Parallel morphological studies were carried out with respect to the mitotic activity and histology of the cultural fragments. To determine mitotic activity, fragments were incubated for 24 hours in a medium containing tritiated thymidine labeled on the methyl group (1 to 2 μc/ml; specific activity, 6.7 c/m*M*), fixed, embedded in paraffin, serially sectioned at 5 μ, and processed through the radioautographic procedure, using the dipping technique (*5*, *7*). Kodak Nuclear Track Emulsion NTB-3 was used, with an exposure time of 10 to 14 days. With every 40th to 50th section of each fragment, an area of 0.16 mm^2 (microscopic field, × 430) showing the greatest degree of labeling was selected, and the number of cells containing ten or more grains per nucleus counted. Counts from four to five fragments for each group of cultures were then averaged to express their mitotic activity. Methyl green–pyronin stain was employed for routine histological examination, and Feulgen's stain used for radioautographs.

Throughout the month of cultivation, the fragments showed excellent preservation of morphology and of functional capacity to produce antibody. The morphological preservation was reflected not only in the maintenance of topographical integrity but also in the multiplication and differentiation of those cells generally considered to participate in antibody formation, for example, hemocytoblasts and immature and mature plasmocytes (*8*). The untreated control cultures often showed a low antibody titer during the first several days, with a subsequent steady decrease in titer to an undetectable level. On the the other hand, cultures that were exposed to the antigen for 2 hours on day 0 responded by producing a classical anamnestic antibody response. The antibody titers were low or undetectable during the first 3 to 6 days, reached a peak for 3 to 9 days between days 9 and 27, and then decreased but remained high for the remainder of the cultivation period (Table 1, Fig. 1). With HCG-sensitized nodes, the minimal concentration that elicited such an anamnestic response in vitro was between 1 and 0.1 IU/ml. The smallest concentration of BSA to stimulate a secondary response has been reported as about 1×10^{-9} g/ml (*9*). When the cultures were treated with PHA instead of the sensitizing antigen under the same conditions, fragments of lymph nodes from four out of seven rabbits responded by producing an anamnestic response (three from five HCG-sensitized rabbits, and one from two rabbits sensitized with BSA). Table 1 shows

Table 1. Titers of antibody to HCG in cultures of HCG-sensitized lymph node fragments following stimulation by HCG (20 IU of Follutein per milliliter) or PHA (600 μg/ml) on day 0; duplicate cultures for each group.

Days in culture	Not stimulated	Stimulated with	
		HCG	PHA
	Reciprocal HCG antibody titer		
0 to 1	40	5	80
	40	5	80
1 to 2	20	40	160
	20	40	160
3 to 4	20	80	160
	20	80	160
4 to 5	20	80	160
	20	80	160
5 to 6	20	40	160
	20	40	160
6 to 7	10	80	160
	10	80	160
7 to 8	5	80	160
	5	80	160
8 to 9	10	160	80
	10	160	80
11 to 14	160	2560	320
	80	2560	160
14 to 17	160	2560	320
	80	2560	160
17 to 20	80	1280	320
	40	1280	80
20 to 23	160	1280	320
	40	1280	80
23 to 26	40	1280	160
	20	640	40

Table 2. Number of cells incorporating tritiated thymidine (per 0.16 mm^2 per day) in cultures of HCG-sensitized lymph node fragments following stimulation by HCG (20 IU of Follutein per milliliter) or PHA (600 μg/ml) on day 0. Average counts of five fragments for each group.

Days in culture	Not stimulated	Stimulated with	
		HCG	PHA
0 to 1	31	82	81
4 to 5	57	208	111
8 to 9	44	142	65

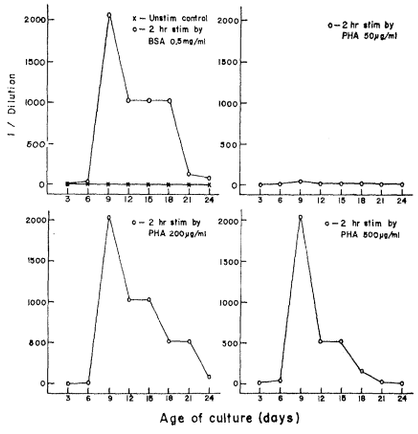

Fig. 1. Reciprocal titers of antibody to BSA in cultures of BSA-sensitized lymph node fragments following stimulation by BSA (0.5 mg/ml) and PHA in concentrations of 50, 200, and 500 μg/ml for 2 hours on day 0. Each point represents the mean value for duplicate cultures.

an example of the stimulation of antibody production and thymidine incorporation in cultures of HCG-sensitized node fragments following treatment with PHA. An increase in the titer of antibody to HCG appeared on the 2nd day, was maintained for 8 days, and then started to decline. The untreated control cultures and cultures stimulated with HCG were used for reference. The apparent shorter latent period and shorter duration of elevated antibody production in PHA-stimulated cultures, as compared to those stimulated with HCG, was not observed consistently when different concentrations of PHA and different rabbits were used. Figure 1 shows the effects of PHA in varying concentrations on the production of antibody to BSA in cultures of BSA-sensitized node fragments. PHA in concentrations of 500 and 200 μg/ml produced an anamnestic response almost exactly like that elicited by BSA, while a PHA concentration of 50 μg/ml showed only a questionable stimulatory effect. Preliminary experiments showed that the effective concentrations of PHA for mitotic stimulation were lower when serum-free medium (*10*) was used.

If the elicitation by PHA of an anamnestic response is truly a nonspecific one, it implies that sensitized cells can transmit to their progeny the necessary information for synthesizing a specific antibody in the absence of exogenous antigen. Thus the antibody titers in the cultures rise because of an increased number of antibody-producing cells resulting from the mitotic stimulation by PHA (Table 2). If this is the case, presence of the antigen is not necessary for the elicitation and maintenance of the anamnestic response.

On the other hand, the elicitation by PHA may be really a specific one in that PHA increases the sensitivity of previously primed cells to traces of antigen still present in the fragments. Normally, these concentrations must be insufficient to produce an anamnestic response, as shown by the negative response in the untreated cultures. If this is the case, PHA may resemble the inducer in the synthesis of induced enzymes (*11*) by increasing the permeability of the cell to the antigen; thus the intracellular antigen may reach a concentration sufficient to trigger synthesis of the antibody (perhaps by derepressing the directing gene). Phytohemagglutinin may also exert its effect by altering the membrane of the sensitized cell so that it is more responsive to the antigen or to whatever compound transfers the information, for example, RNA (*12*), or RNA-antigen complex. The effectiveness and persistence of the PHA stimulatory effect after such short contact raises the possibility that PHA may act on the level of the cell membrane; conceivably, for the same reason, so may the antigen. It is difficult at present to explain the three experiments in which the sensitized cells did not respond to PHA, but did respond to the specific antigen by producing an anamnestic response.

Elicitation by PHA of an anamnestic response in vitro thus provides a different approach for investigating the role of antigen in antibody formation.

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References and Notes

1. G. Dreyer and E. W. A. Walker, *J. Pathol. Bacteriol.* **14**, 28 (1909).
2. F. J. Dixon and P. H. Maurer, *J. Immunol.* **74**, 418 (1955).
3. Phytohemagglutinin was obtained from Difco; bovine plasma albumin, from Armour.
4. Follutein, E. R. Squibb & Sons.
5. T. W. Tao, thesis, Harvard Univ. (1962).
6. A. B. Stavitsky, *J. Immunol.* **72**, 360 (1954).
7. D. L. Joftes and S. Warren, *J. Biol. Phot. Assoc.* **23**, 145 (1955).
8. A. H. Coons, *J. Cellular Comp. Physiol.* **52** (Suppl. 1), 55 (1958).
9. T. F. O'Brien, M. C. Michaelides, A. H. Coons, *J. Exptl. Med.* **117**, 1053 (1963).
10. C. T. Ambrose, *ibid.* **119**, 1027 (1964).
11. A. Novick and M. Wiener, *Proc. Natl. Acad. Sci. U.S.* **43**, 553 (1957); J. Monod, in *Cellular and Humoral Aspects of the Hypersensitive States*, H. S. Lawrence, Ed. (Harper, New York, 1959), p. 628.
12. J. A. Mannick and R. H. Egdahl, *Science* **137**, 976 (1962); M. Fishman, R. A. Hammerstrom, V. P. Bond, *Nature* **198**, 549 (1963).
13. This study was supported by USPHS research grant HD-00137. I thank Prof. A. T. Hertig for his generous support and Prof. A. H. Coons for his valuable criticism and suggestions regarding the manuscript. To C. T. Ambrose, I owe gratitude for encouragement and assistance.
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Chromosome Abnormalities in vitro in Human Leukocytes Associated with Schmidt-Ruppin Rous Sarcoma Virus

Abstract. *Chromosome breakage was observed in human leukocytes in vitro when Schmidt-Ruppin strain of Rous sarcoma virus was added to the cultures. Similar additions of Bryan strain of Rous sarcoma virus had no such effect.*

The Schmidt-Ruppin strain of the Rous sarcoma virus produces tumors in a wide variety of mammals as well as in fowl (*1*). Affected mammals include the mouse, rat, Syrian hamster, rabbit, and guinea pig. A similar strain of the Rous sarcoma virus (Zilber) produces tumors in primates (*2*). We have previously studied the effect of the Schmidt-Ruppin virus on chromosomes in the cells of rat tumors induced by the virus, in the cells of similar tumors in tissue culture, and in diploid rat embryo cells in tissue culture (*3*). In all three systems chromosome breaks, or rearrangements resulting from breaks, occurred. Because of the wide variety of mammals in which this virus produces tumors and the association of the virus with chromosome breakage, its effect was studied in a human cell system in vitro. The cells, human peripheral leukocytes, were chosen primarily because in culture they are induced to divide by addition of phytohemagglutinin (*4*); therefore the times of RNA and DNA synthesis and the state of mitosis when virus is added are known with relative accuracy (*5*).

Pools of cell-free virus suspension were prepared by harvesting rapidly growing tumors induced in the chicken. The tumors were minced and put in suspension (10 percent by weight) in tris-saline solution containing 10 per-