Transformation of Rabbit Lymphocytes by Specific Antigens.* (32342)

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(Introduced by B. H. Waksman)
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The reactions of peripheral lymphocytes *in vitro* to immunologically specific and non specific stimulants have been intensively investigated during the last decade(1,2). Most of this effort, however, has been focused on human cells and few investigations have dealt with lymphocytes from experimental animals. Earlier unsuccessful attempts to obtain a specific reaction toward immunizing antigens(3,4) have been followed by 3 very recent reports of transformation of guinea pig and rabbit lymphocytes by specific antigens (5,6,7).

We record here some preliminary findings concerning the specific reaction of rabbit lymphocytes to the different antigens against which the animals were sensitized.

Materials and methods. Antigens. Bovine and rat heart homogenates were prepared as described by Gery and Davies(8). The extract supernates for immunization were obtained by centrifugation at 12,000 g for 30 minutes; for stimulation of lymphocyte cultures they were centrifuged at 25,000 g for 60 minutes. Human IgG was prepared according to the procedure of Campbell *et al*(9).

Immunization. Male or female mongrel rabbits weighing 2-4 kg were injected intradermally and into the hind foot pads with antigen emulsions in complete Freund's adjuvant (Difco), in a total volume of 1.0 ml. Inital injections contained 10 mg of human IgG or 20 mg (protein) of heart extracts. One month later, a booster dose of 10 mg antigen without adjuvant, was given subcutaneously and intramuscularly. Rabbits injected with adjuvant alone or untreated, served as controls.

Lymphocyte cultures. Blood samples were drawn from the central artery of the ear or the heart, 3 weeks after the first injection or 1-4 weeks after the booster. Heparin (50-100 units/ml) was added to each blood sample and the sedimentation of erythrocytes was enhanced by adding 1/3 volume of 3.5% dextran (Pharmacia, T-250). The leukocyte enriched plasma was incubated for 60 minutes in flat walled bottles (to remove phagocytes) and then spun down. The cell sediment was resuspended in M-199 medium and aliquots of 1.2 to 1.5 $\times$ 10^6 cells were inoculated into rubber stoppered glass tubes with addition of stimulants, 20% allogeneic or autologous serum and M-199 medium to give 2.0 ml final volume. As stimulants, antigens in different concentrations, or staphylococcal filtrate, (0.5 ml/culture, prepared according to Ling and Husband(10)) were used. After 5 days' incubation at 37° the cells were fixed and stained by the method of Bach and Hirschhorn (11). Blast cells and mitoses were counted in a total of 400-1000 cells. Colchicine was not added.

H^3-thymidine incorporation. On the fourth day of incubation, 1 μc of methyl tritiated thymidine (Schwarz BioResearch Inc., 1.9 c/m mole) was added to each culture. Twenty-four hours later, the cells were spun down, haemolyzed by 1% citric acid, washed twice with M-199 medium and resuspended in 3% normal serum (in medium) to which trichloroacetic acid (TCA) was then added to 5% final concentration. The precipitate was collected on Millipore filters (pore size, 0.45 μ) and the radiation counted in a Packard Tricarb Liquid Scintillation Spectrometer.

Results. The percentages of blast formation and mitoses induced in cultures of lymphocytes from immunized rabbits are summarized in Table I. All rabbits tested showed an intensive reaction, of both blast transformation and mitotic activity, to staphylococcal filtrate. High responses (up to 51.5% blasts) were found also in cultures to which the immunizing antigen was added (Fig. 1) and these responses always significantly higher than those found in control cultures. Both kinds of antigen, soluble protein (human IgG) and tissue

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TABLE I. Stimulation of Blast Transformation and Mitoses in Rabbit Lymphocyte Cultures.

Rabbit	Immunizing antigen	*In vitro* stimulants: Staph. filtrate	Bovine heart	Rat heart	Human IgG	None (control)
T-1	Bovine heart	—*	40.2 (1.2)†	23.8 (.4)	—	3.6 (.0)
T-3	" "	81.4 (.9)	44.8 (.6)	8.8 (.2)	—	.4 (.0)
T-4	" "	78.6 (1.7)	51.5 (.8)	19.9 (.2)	—	23.5 (.0)
T-10	Rat heart	67.6 (1.5)	—	47.2 (.8)	20.8 (.2)	23.9 (.1)
T-11	" "	70.0 (1.1)	—	52.0 (1.1)	13.8 (.0)	5.0 (.0)
S-1	Human IgG	58.7 (.5)	—	—	29.3 (.5)	12.7 (.0)
S-2	" "	75.8 (.6)	—	—	42.2 (.9)	20.2 (.0)
A-2	Complete adjuvant	70.5 (1.2)	8.2 (.0)	6.9 (.0)	—	8.7 (.0)
N-5	None (normal)	63.0 (.9)	7.8 (.0)	5.2 (.0)	—	10.6 (.0)
N-6	" "	74.9 (.5)	4.3 (.0)	7.8 (.0)	5.3 (.0)	4.5 (.0)

* Not done.
† Percent blast cells in culture; percent of mitoses in parentheses.

extracts (bovine or rat heart) were found to be potent inducers of the lymphocyte response. Reactions of cross reacting antigens were, sometimes, though not always, higher than those of the controls.

The control cultures showed a high degree of variation in the number of blast cells after the 5 days of incubation. The percentage of blasts ranged from 0.4% to 23.9% and high counts were found in cultures of both normal and immunized rabbits. The proportion of blast forms was not decreased by use of autologous rather than allogeneic serum. In contradiction to this marked blast formation, few or no mitoses were found in control culture while the reactions to the stimulants, both staphylococcal filtrate and specific antigen, was accompanied by a clear mitotic response.

A sharper indication of the *in vitro* responses of lymphocytes was achieved by determination of H^3-thymidine uptake by the cells in culture (Table II). In all cases, significant incorporation of thymidine was shown by stimulated cultures, in distinction to minimal uptake by the cells in control tubes. Little thymidine incorporation was found in control cultures even when blast formation was con-

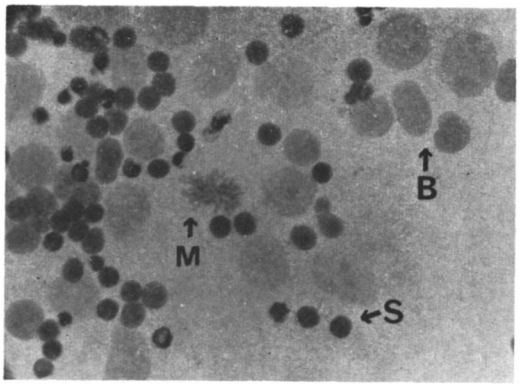

FIG. 1. Reaction of peripheral lymphocytes from rabbit immunized with beef heart to the specific antigen. Orcein acetic acid staining. B = blast cell; M = mitosis; s = small lymphocyte.

TABLE II. H^3-Thymidine Incorporation and Morphological Changes of Rabbit Lymphocytes.

Rabbit	Immunizing antigen	*In vitro* stimulant	H^3-thymidine uptake (cpm × 5)	% Transformation: Blasts	(Mitoses)
T-3	Beef heart	Staph. filtrate	54,510	61.3	(1.0)
		Beef heart	61,042	42.1	(.9)
		Human IgG	129	1.9	(.0)
		None (control)	184	1.0	(.0)
T-10	Rat heart	Staph. filtrate	72,117	80.0	(1.5)
		Rat heart	46,900	42.7	(.3)
		None (control)	1,400	25.6	(.0)
S-1	Human IgG	Staph. filtrate	22,356	58.7	(.5)
		Human IgG	3,335	29.3	(.5)
		None (control)	1,340	12.7	(.0)

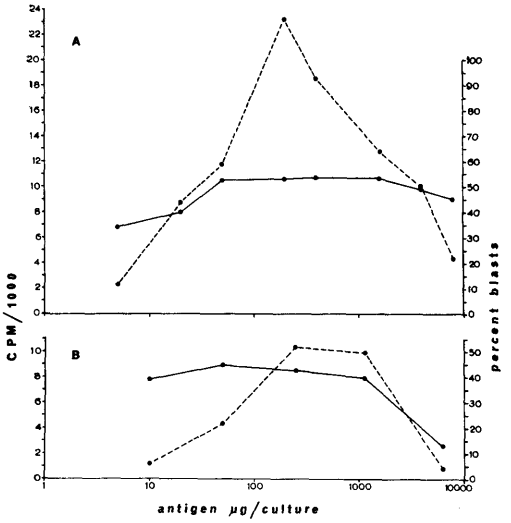

FIG. 2. Effect of antigen concentration on blast transformation and H^3-thymidine uptake by rabbit lymphocytes. Rabbits were immunized against beef heart (A) or rat heart (B) and the lymphocytes stimulated *in vitro* by the corresponding extract. (— — —) H^3 -thymidine incorporation; (———) percent blast cells.

siderable such as in T-10 and S-1. The thymidine incorporation induced by the different stimulants paralleled the morphological response.

The effect of antigen concentration in the culture on the cell response is shown in Fig. 2. Maximum reactions were produced by antigen at concentrations of 100-300 μg/culture. Smaller amounts of antigen gave a lower response and excess antigen also led to a decreased response.

Lymphocytes collected at the different times after immunization reacted similarly to the specific antigens.

Discussion. Lymphocytes cultured from peripheral blood of immunized rabbits reacted strongly to the specific antigens *in vitro*. The significance of the reaction was best demonstrated by thymidine incorporation and mitotic activity which were not found in control cultures. A very high degree of blast transformation was found in the stimulated cultures, although, in some cases, considerable values were also manifested by control lymphocytes.

A high "spontaneous" blast formation in control cultures of rabbit lymphocytes was reported by Sabesin(12) and Fikrig *et al*(13) to occur after 5 days of incubation. The values cited by these authors were, however, higher than ours and occurred in all cultures, while others(4) failed to find such spontaneous reactions. These variations may be attributed both to differences in culture techniques and to differences in behavior of different strains of rabbits. Our findings give some support to the latter possibility: great variations were found among the experimental rabbits, with some animals giving constantly high values of blast formation and others always lacking this spontaneous reaction.

The degree of blast formation stimulated in our experiments by specific antigens exceeds by far the reactions reported with lymphocytes of immunized guinea pigs(6). The difference may be attributed to variations in method, mainly in regard to bleeding schedule and immunizing dose, while technical factors may also explain the failure of earlier attempts (3,4) to obtain a response *in vitro*.

The mitotic activity found in our cultures was low as colchicine was not added. Similar values were found by Zweiman *et al*(5) in guinea pig lymphocyte cultures stimulated by old tuberculin, with, however, the addition of colchicine. The levels of thymidine incorporation by the stimulated cells in the present experiments resemble those reported by Vischer *et al*(7) with rabbit lymphocytes.

The kind of immunity involved in the *in vitro* response of the lymphocytes could not be determined in the present experiments, since all immunized rabbits manifested a vigourous response, both of serum antibody production and delayed skin reactivity. Moreover, the addition of antibody containing autologous serum did not affect the response, a finding contrary to that of Heilman and McFarland(14) who reported that serum antibodies inhibit the *in vitro* reaction of lymphocytes from donors hypersensitive to tuberculin. In other experiments (unpublished), lymphocytes from rabbits immunized without adjuvant showed a striking reaction to antigen; these rabbits gave very little or no delayed response.

Increasing concentrations of antigen increase the degree of *in vitro* response up to an

optimum, after which, excess antigen leads to a decrease in the cell reaction. A similar relationship was shown with lymph node cells by Oppenheim *et al*(6). The reduction of response *in vitro* by high doses of antigen resembles the induction of unresponsiveness in immunized animals(15,16), but may as well be due to toxic effects.

The mechanisms involved in the response of the rabbit lymphocyte *in vitro* and its relationship to other immune reactions are under investigation.

Summary. Lymphocytes from the blood of rabbits immunized with extracts of bovine or rat heart or with human IgG, reacted *in vitro* when cultured with the specific antigen. The reaction consisted of intense blast transformation, mitotic activity and incorporation of H^3-thymidine. The level of response was found to be dose-dependent. Some unstimulated (control) cultures showed moderate "spontaneous" blast formation, without, however, mitotic activity or thymidine uptake.

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Relationship of Life Maintaining Properties of Corticoids to Their Influence on Compensatory Ovarian Hypertrophy in Hemicastrated-Adrenalectomized Female Rats. (32343)

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Hypertrophy of the remaining ovary after hemicastration is a well-known phenomenon. Although the mechanism of the hypertrophy remains to be elucidated, we believe that removal of one ovary results in increased gonadotrophin secretion (synthesis and/or release) by the pituitary gland(1) which produces growth of the ovary. Adrenalectomy, at the time of unilateral ovariectomy, will abolish the ovarian response, while adrenal replacement therapy with glucocorticoids or mineralocorticoids or saline support will restore the ovarian response(2). At the original corticoid doses employed (cortisol, 1000 μg/day or DOC, 500 μg/day) most animals survived the 2-week experimental period, whereas mortality was high in the untreated groups. In an effort to determine the relationship of corticoid normalization of the ovarian response to their life maintaining effects, we have now examined series of doses of desoxycorticosterone and corticosterone in hemicastrated-adrenalectomized rats.

Materials and methods. Adult (160-180 g) female Charles River CD® rats were adrenalectomized and hemicastrated by removal of the right ovary. Drug treatment began immediately after operation and continued for