THE STIMULATION OF DNA SYNTHESIS IN CULTURES OF RABBIT LYMPH NODE AND SPLEEN CELL SUSPENSIONS BY HOMOLOGOUS CELLS*,‡

By NILES D. CHAPMAN,§, || M.D., and RICHARD W. DUTTON, Ph.D.

(From the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California)

Plate 1

(Received for publication, August 24, 1964)

Previous communications have shown that the addition of homologous antigen to suspensions of spleen or lymph node cells from immunized rabbits stimulates the incorporation of thymidine in the DNA of 1 to 2 per cent of the cell population (1–3). The cells involved are principally large undifferentiated cells which undergo cell division (2). It has been shown that cultures stimulated under these conditions will go on to synthesize antibody (4). This response has been taken to represent the *in vitro* counterpart of the cellular proliferation seen in the secondary immune response *in vivo*, and the experimental model has been used in studies of the mechanism of this response (5–9).

In the course of these studies it was observed that a similar proliferative response occurred when spleen or lymph node suspensions of two unrelated (homologous) rabbits were incubated together. This response would appear to be similar to that noted in mixed cultures of human peripheral leukocytes described earlier by Bain *et al.* (10, 11) and Hirschhorn *et al.* (12).

Bain *et al.* (10, 11) made 1:1 mixtures of suspensions of human peripheral leukocytes (from non-related subjects) in heparinized plasma and incubated these and control cultures at 37°C for 5 days. Inspection of stained smears of the mixed cultures revealed a considerable number of very large cells with basophilic cytoplasm which were absent in the controls. Some of the large cells were in mitosis. It was further found that if such cultures were incubated at 5 days with tritiated thymidine for 1 hour, from 4 to 45 cells/1000 became labeled in the mixed cultures compared with 1

* This is publication number 88 from the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla.

‡ This work was supported in part by a grant from the United States Public Health Service and in part by a Dernham Scholarship of the California Division, American Cancer Society (number D-100) from Scripps Clinic and Research Foundation, La Jolla.

§ Supported by a United States Public Health Service training grant GM-325-05 from the Department of Surgery, University of Washington School of Medicine, Seattle, Washington.

|| Present address: Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla.

to 9/1000 in the controls. No reaction was noted when the peripheral leukocytes of monozygotic twins were mixed and no response was obtained to homologous erythrocytes.

Hirschhorn and his colleagues (12, 13) have described similar findings. In their experiments, as many as 50 to 70 per cent of the cells were large "transformed" cells in mixed cultures observed at 4 and 7 days, but there is no clear indication whether these cells represent the transformation of a major fraction of the original cells or are the progeny of a much smaller fraction stimulated to rapid proliferation. They have used this effect as the basis for a suggested histocompatibility test (13, 14). Extracts of homologous leukocytes have also been shown to be stimulatory (15, 16).

These and other workers have demonstrated similar *in vitro* changes in peripheral leukocytes brought about by antigens to which the subject had been immunized (17–26), by extracts of autologous tissues in patients with autoimmune diseases (15, 26, 27), antisera from rabbits immunized to human leukocytes (28, 29), and by phytohemagglutinin M (12, 25, 30–37). The response to the latter was found to be most intense.

It is the purpose of this report to describe our studies of the response of mixtures of lymphoid cells from unrelated rabbits and to discuss its significance and its relation to other analogous phenomena. A preliminary account of this work has already appeared (38).

Materials and Methods

Animals.—New Zealand white male rabbits weighing 2 to 4 kg were all obtained from the same outbred but closed stock.

Immunization.—In some experiments, animals were immunized by a 4 week course of 16 intravenous injections of alum-precipitated bovine or human albumin or globulin.

In other experiments, recipient rabbits were immunized against individual homologous donor rabbit tissues either by single 4 x 3 cm skin grafts 25 to 27 days before the *in vitro* test or by single intraperitoneal injection of a suspension prepared from whole donor spleens. All the cells from one donor spleen were injected into a single recipient. The spleens for these injections were removed utilizing sterile surgical technique. The donor rabbit was sacrificed later and the mesenteric nodes used in the *in vitro* experiment.

Cell Suspensions.—Cell suspensions were prepared from spleen, mesenteric and popliteal lymph nodes, bone marrow, thymus, and alveolar macrophages, as previously described (2, 3). The spleens were perfused with 5 ml of phosphate-buffered saline containing 1 per cent normal rabbit serum before removal to reduce red cell contamination. The solid tissues were lightly minced with scissors, sieved, and washed 3 times in phosphate-buffered saline containing 1 per cent normal rabbit serum. The cells were finally suspended in Eagle's minimum essential (MEM)[1] suspension medium supplemented with non-essential amino acids, glutamine, sodium pyruvate, penicillin and streptomycin, and 15 per cent normal rabbit serum.[1]

Cell suspensions were filtered through sterile nylon gauze. It had previously been shown that the rate of DNA synthesis measured by the technique to be described was dependent on the density of the cell suspensions (39). It was therefore necessary to ensure that the total cell density of all the cultures compared was the same. All cell suspensions were, therefore,

[1] The Eagle's MEM suspension medium and supplement concentrates were obtained from Microbiological Associates, Bethesda. Normal rabbit serum was obtained from Pel-Freez Biologicals, Incorporated, Rogers, Arkansas (blood serum, type 3) and filtered sterile.

adjusted to 1.5 × 10^7 cells/ml before mixing and all mixtures consisted of a 50:50 mixture of the 2 suspensions unless otherwise described. All cultures, single or mixed, therefore, contained the same total number of cells in the same volume of medium. The suspensions were incubated at 37°C in an atmosphere of 5 per cent CO_2 in 95 per cent O_2.

Measurement of the Rate of DNA Synthesis.—The rate of DNA synthesis in cell suspensions was measured by the uptake of tritiated thymidine, as previously described (reference 2 as modified in reference 6). Five-tenths ml of medium containing 2 μc H^3 thymidine with a specific activity of 80 mc/mmole were added to 1.5 ml of the cell suspension at various times after the start of the incubation period. The cells were harvested after further standard incubation periods of 6 or 12 hours.

In other experiments where the time course of the rate of DNA synthesis was not followed, the thymidine was added at 24 hours and the cultures harvested at 48 hours. The harvested cells were washed once in ice-cold saline, twice in ice-cold 5 per cent TCA and twice in ice-cold methanol. The cells were then dissolved in 1 ml of "hyamine* 10-X" and counted in a liquid scintillation counter. It has been shown that more than 95 per cent of the incorporated counts can be recovered as isolated DNA (2), and the incorporation of radioactivity into washed cells has been taken to be a reliable measure of DNA synthesis. The standard deviation of the values obtained from replicate cultures was less than 15 per cent. The results reported represent the mean values of 3 to 4 replicate cultures.

Autoradiographs.—Autoradiographs were prepared from cultures of cells that had been incubated for 1 hour with 12 μc of H^3 thymidine with a specific activity of 240 mc/mmole. Following this, the cells were harvested and washed twice with phosphate-buffered saline. If these cells were further cultured, non-radioactive thymidine was added to the culture medium to minimize the uptake of any tritiated thymidine that might possibly remain. At the end of the second culture period, the cells were again washed, well drained, and resuspended in 1 drop of normal rabbit serum. They were transferred to microscope slides with a camel hair paint brush. The preparations were rapidly dried in a stream of air at room temperature and fixed in methyl alcohol for 15 minutes. Autoradiographs were made with Kodak NTB2 emulsion and exposed for 5 to 10 days. Slides were developed in Kodak D19B developer and stained with Giemsa stain.

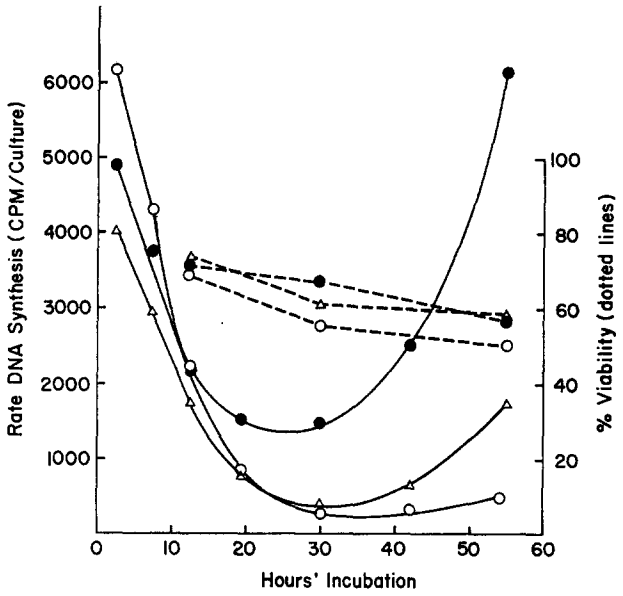
RESULTS

The rate of thymidine incorporation (DNA synthesis) into cells of freshly isolated spleen, lymph node, thymus, or bone marrow suspensions falls rapidly during the first 24 hours and then becomes relatively constant. The cell viability, determined by the exclusion of trypan blue, falls from 95 per cent to 50 to 70 per cent during this period. DNA synthesis in spleen and lymph node suspensions from immunized rabbits is stimulated by the addition of the immunizing antigen *in vitro* (2, 3). Thymus, bone marrow, and alveolar macrophage suspensions fail to show such a response.

There was a marked stimulation of DNA synthesis when lymph node or spleen cell suspensions of 2 unrelated rabbits were incubated together (Text-fig. 1). The stimulation of DNA synthesis became apparent by 24 hours. There was no evidence of any difference in the viability of the cells in separate or mixed cultures. The time course and magnitude of the stimulation of DNA synthesis in the "mixed" cultures was found to be comparable to that seen in a secondary immune response as measured by this system (Text-fig. 2). Since

the response became clearly apparent during the second 24 hours' incubation, measurement of the uptake of radioactive thymidine during the period 24 to 48 hours was adopted as the standard procedure in subsequent experiments. There was a considerable variation in the size of the response from experiment to experiment, but in very few instances was there no response at all.

In some experiments, lymphoid cell suspensions from unrelated rabbits



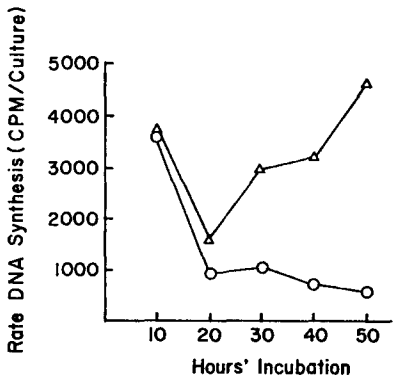
TEXT-FIG. 1. Stimulation of DNA synthesis in homologous mixtures of lymph node suspensions. Suspensions of lymph node cells from two normal rabbits were incubated alone, L1, ○, L2, △, or in a 50:50 mixture, L1 + L2, ●, containing the same total number of cells as the separate suspensions. The rate of DNA synthesis was measured at various times by the uptake of radioactive thymidine during a standard incubation period. Viability was determined by counting the per cent of cells that did not stain with trypan blue.

which had been previously immunized to heterologous serum proteins were used. The response of these cell suspensions to homologous cells, to antigen, and to the simultaneous presence of antigen and homologous cells was measured. Simultaneous responses of cells from immunized rabbits to the immunizing antigen and to homologous cells appeared to occur independently as the responses to both mixing and antigen was, in general, greater than either alone (Table I).

Spleen or lymph node cells appeared to be capable of initiating the proliferative response when exposed to cultures of homologous spleen and lymph node cells (Table II). Irradiation (400 roentgens) of one of the partners in a homol-

ogous mixture prevented these cells from making a response. Irradiated cells, however, still functioned as a stimulus to the unirradiated partner (Text-fig. 3.)

No stimulation was observed when thymus cells suspensions from two animals were mixed and it was, therefore, concluded that the thymus cells could



TEXT-FIG. 2. Antigenic stimulation of DNA synthesis in spleen cell suspensions from an immunized rabbit (Fig. 1 of reference 2). The antigen was added at zero time and the rate of DNA synthesis determined by the uptake of radioactive thymidine during a standard incubation period. △, antigen; ○, control.

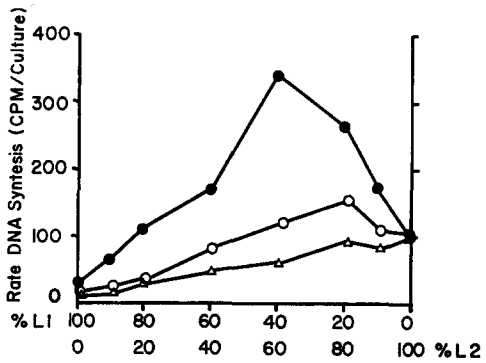
TABLE I

Simultaneous Response of Immunized Lymphoid Cell Cultures to Antigen and to Homologous Cells

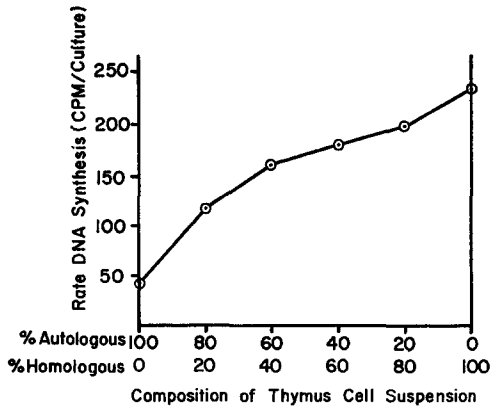
Cell suspensions	Experiment No.			
	220	211	259	404
1. R1	49	505	248	4
2. R1 + antigen	103	2106	907	6
3. Calculated excess due to antigen (2 − 1)	(54)	(1601)	(659)	(2)
4. R2	50	442	226	39
5. R2 + antigen	174	2734	846	185
6. Calculated excess due to antigen (5 − 4)	(124)	(2292)	(620)	(146)
7. R1 + R2	225	1594	1248	1899
8. Calculated excess due to "mixing" 7 − (1 + 4)/2	(175)	(1320)	(1011)	(1877)
9. R1 + R2 + antigen	309	2360	1521	2567

Cell suspensions from two rabbits, R1 and R2, immunized to the same antigen were incubated for 48 hours alone or mixed, each in the presence or absence of antigen. The rate of DNA synthesis was measured by the uptake of thymidine during the period 24 to 48 hours. The results of four separate experiments are presented. Calculated values for the stimulation due to antigen and due to mixing are shown in parentheses.

not themselves take part in the response (Table II). That thymus cells are, at times, capable of exerting a stimulatory effect is clearly shown in the experiment illustrated in Text-fig. 4 where lymph node cells were exposed to varying numbers of homologous thymus cells diluted out in a suspension of autologous



TEXT-FIG. 3. Stimulation of DNA synthesis in homologous mixtures of lymph node suspensions. Effect of irradiation. Mixtures containing varying proportions of normal and irradiated lymph node suspensions from two animals were incubated for 48 hours. The rate of DNA synthesis was measured by the uptake of radioactive thymidine over the period 24 to 48 hours. Mixtures of L1 + L2, ●; mixtures of irradiated L1 and non-irradiated L2, ○; mixtures of irradiated L2 and non-irradiated L2, △. The latter mixture served as a control. The relative proportions of L1, irradiated L1, or irradiated L2 *versus* L2 are indicated on the horizontal axis. In the absence of stimulation all intermediate points lie on the straight line joining the two extremes as in irradiated L2 mixed with L2.



TEXT-FIG. 4. Stimulation of DNA synthesis in normal lymph node by homologous thymus cells. The cell suspensions consisted of 50 per cent normal lymph node cells and 50 per cent of mixtures of homologous and autologous thymus cells. The relative proportions of homologous and autologous thymus cells are indicated on the horizontal axis. The mixed suspensions were incubated for 48 hours and the rate of DNA synthesis measured by the uptake of thymidine over the period 24 to 48 hours.

thymus cells. However, thymus cells were not as strong or consistent in exerting a stimulatory effect as other lymphoid cells (Table II). Autologous mixtures of spleen, lymph node, and thymus cells showed no stimulation of DNA synthesis (Table II).

TABLE II

The Rate of DNA Synthesis in Separate, Homologous Mixed and Autologous Mixed Suspensions

Separate suspensions

	Rabbit 1	Rabbit 2
Spleen (S)	505	442
Lymph node (L)	61	33
Thymus (T)	676	348

Mixed suspensions

Homologous				Autologous			
	Observed	Calc.	Ratio		Observed	Calc.	Ratio
S1 + S2	1594	478	3.4	L1 + T1	182	369	0.5
L1 + L2	357	47	7.6	S1 + L1	356	283	1.3
S1 + L2	934	269	3.5	S1 + T1	452	591	0.8
S2 + L1	481	252	1.9	S2 + L2	151	238	0.6
T1 + T2	514	512	1.0	S2 + T2	178	395	0.5
S1 + T2	299	427	0.7	L2 + T2	63	191	0.3
S2 + T1	727	559	1.3				
L1 + T2	164	205	0.8				
L2 + T1	481	355	1.35				

The cell suspensions indicated were incubated for 48 hours. All suspensions contained the same total number of cells. Mixed suspensions were mixed in the ratio 1:1. The rate of DNA synthesis was measured in the period 24 to 48 hours. The figures in the three columns for mixed suspensions represent the observed rate of DNA synthesis, the rate calculated as the arithmetic mean of the results obtained from the separate suspensions, and the ratio of the observed to calculated figures.

Bone Marrow.—Initial rates of thymidine incorporation into suspensions of bone marrow cells were very high, presumably reflecting the myeloid activity of such cells (3). There was a rapid decline in the rate of incorporation on incubation and no stimulation of DNA synthesis could be observed in homologous mixed cell suspensions.

The addition of macrophages to spleen or lymph node cells of the same animal resulted in a marked suppression of DNA synthesis (40) and a similar effect was noted in homologous mixes of macrophages. Homologous erythro-

cyte suspensions did not stimulate DNA synthesis in spleen or lymph node cultures (Table III).

Microscopic examination of brush smears of homologous mixed cultures prepared at 0 to 48 hours after mixing revealed no evidence of any cellular interaction. The viability and general appearance of mixed cultures was not measurably different from that observed in the "pure" cultures. Twenty to 50 per cent of the cells in a suspension of lymphoid cells from a single rabbit

TABLE III

The Effect of Homologous Erythrocytes on DNA Synthesis in Spleen or Lymph Node Suspensions

	Autologous RBC	Homologous RBC	
Experiment 215			
Spleen	432	454	
Lymph node	127	104	
Experiment 220			
Spleen 1	225	352	(Spleen 1 + Spleen 2, 4055)
Lymph node 1	124	125	
Spleen 2	2445	2779	(Lymph node 1 + Lymph
Lymph node 2	67	57	node 2, 225)

Spleen or lymph node suspensions (1.5×10^7 cells per culture) were incubated for 48 hours in the presence of autologous or homologous erythrocytes (0.5×10^7 cells per culture). The rate of DNA synthesis was measured by the uptake of radioactive thymidine over the period 24 to 48 hours. In Experiment 220, two rabbits were used, each being the source of homologous erythrocytes for the other. That lymphoid cells of these two rabbits were capable of a response to each other is shown by the figures (in parentheses) for a spleen cell mix and a lymph node mix in the right-hand column.

became stainable with trypan blue by 48 hours and many cells seen in stained preparations made at this time showed degenerative changes.

Autoradiographic studies revealed that only 1 to 4 per cent of the cells in the mixed cell suspensions incorporated thymidine. The labeled cells were predominantly large undifferentiated cells with thin rims of faintly staining cytoplasm. Occasionally more differentiated cells with eccentric nuclei and more deeply staining cytoplasm were seen. A typical cell is illustrated in Fig. 1.

In one experiment pure and mixed cell suspensions were pulse labeled with tritiated thymidine for 1 hour. Preparations were made at this time and replicate cultures were washed and reincubated for a further 28 hours in the absence of any labeled thymidine. The increase in the number of labeled cells in the mixed culture during the second incubation period demonstrated that the increased rate of DNA synthesis was accompanied by cell division (Table IV).

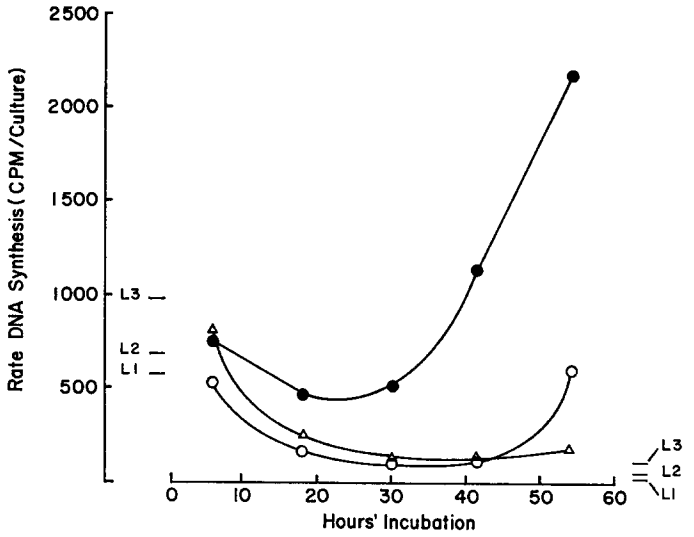
In further experiments it was demonstrated that direct contact between the two cell suspensions was necessary for the response to occur. When cell suspensions from the two animals were separated by a Millipore membrane (average pore size 0.45μ), no stimulation occurred.

TABLE IV

Thymidine Incorporation and Cell Division in Homologous Mixtures of Lymph Node Suspensions

	Cells labeled at 20 hours	Cells labeled at 48 hours
	per cent	*per cent*
L1	0.1	0.1
L2	0.3	0.4
L1 + L2	2.0	4.0

Replicate lymph node suspensions of two rabbits (L1 and L2) were incubated for 20 or 48 hours, either separately or mixed. Twelve microcuries of tritiated thymidine at 240 mc/mmole were added at 19 hours and the cultures harvested after 1 hour. Smears were prepared and autoradiographs were made of each of the three suspensions as described in the methods section. Other replicates were washed, resuspended in medium containing unlabeled thymidine (6 μg/ml), and incubated for a further 28 hours. At the end of this time the suspensions were again harvested and the autoradiographs prepared.



TEXT-FIG. 5. The time course for the rate of DNA synthesis was measured for three pairs of mixed lymph node suspensions: L1 + L2, ○; L1 + L3, ●; and L2 + L3, △. L2 cells were obtained from rabbit 2, the "recipient," which had previously rejected a skin graft from rabbit 1, the "donor." L1 cells thus came from the donor rabbit and L3 cells came from a third rabbit, the "normal" which was neither donor nor recipient. The results show that the response for the donor-recipient combination (L1 + L2) was lower than that for the donor-normal combination and higher than the recipient-normal combination.

Preimmunization of Lymph Node Donors.—A skin graft was taken from rabbit 1 (donor) and placed onto rabbit 2 (recipient). A third rabbit was used as a control (rabbit 3 normal). The grafts were made 25 to 27 days before the rabbits were sacrificed. Time curves for the rates of DNA synthesis were then determined on the three possible homologous mixtures of spleen or mesenteric lymph node suspensions, donor plus normal, recipient plus normal, donor plus recipient. There was no evidence in two such experiments that prior exposure of the "recipient" to "donor" skin graft enhanced the response in a donor-recipient "mix" relative to the other two (Text-fig. 5), since, in each case, the greatest response was obtained with a non-immunized mix. Since it might be held that the method for sensitization of the recipient was inadequate, the experiment was repeated using an intraperitoneal injection of spleen cells from the donor rabbit to immunize the recipient. Again, there was no evidence of a heightened response when donor and recipient lymph node cell suspensions were mixed. However, in view of the large variation in the size of responses obtained in non-immumized mixes, it cannot be stated categorically that enhancement had not occurred.

DISCUSSION

The response described here would appear to be analogous to the findings of Bain *et al.* (10, 11) and Hirschhorn and his colleagues (12) on the mixing of cultures of human peripheral leukocytes.

The present studies extend their observations and establish several further points. They also exhibit some important differences and raise the problem of the interpretation of the significance of the response.

In the work described, an early proliferative response follows the mixing of certain lymphoid tissues from two unrelated rabbits. Only spleen and lymphoid cells appear able to respond to homologous tissue. The thymus may on occasion stimulate the response, but is incapable itself of responding to stimulation by homologous tissue. This is in keeping with the immunologic potentiality of this tissue as demonstrated by other techniques. Homologous erythrocytes or autologous tissues do not stimulate a response. It is not possible to comment on the results of mixing homologous alveolar macrophages or bone marrow cells with spleen or lymph node cells, as any stimulatory effect that may have occurred is obscured by the operation of other factors. The response does not occur when the 2 cell suspensions are separated by a 0.45 μ Millipore membrane. Actual contact with a homologous cell rather than with a secreted product would appear to be necessary.

It was of considerable interest that simultaneous additive responses could be obtained to homologous cells and to antigen when cell suspensions from immunized rabbits were used. It is possible that different populations of cells are involved in the two responses. It is also clear that the interaction of the two

homologous cell populations does not interfere with their function, since each does not appear to be markedly hindered in its response to antigen, at least during the 48 hour period of observation.

The increase in the rate of DNA synthesis begins very soon after mixing and is clearly measurable by 6 to 20 hours. The increase in the number of cells incorporating thymidine at later times (48 hours) is largely, if not solely, due to the proliferation of a small population (1 to 2 per cent) of responding cells. These are large undifferentiated cells at the time when they can first be identified as responders (20 hours). The present studies provide no evidence as to whether or not these cells do arise by the transformation of small lymphocytes during the first 20 hours.

In Hirschhorn's studies, it was claimed that up to 80 per cent of the cells have transformed into proliferating blast cells by 8 days. It is thus implied that the majority of the cells present initially take part in the response but it is in fact not clear from his data what proportion of the initial cells are the progenitors of these "transformed" cells.

It would seem quite possible from the present studies that the large numbers of transformed cells seen by Hirschhorn at 8 days could have arisen by the rapid proliferation of perhaps as little as 1 to 2 per cent of the initial population.

It was a remarkable finding that the speed and magnitude of the response are in every way comparable with the secondary response of cell suspensions from immunized rabbits exposed to the immunizing antigen. Primary responses to heterologous serum proteins, as antigen, are not observable with this experimental model presumably because the cell population that is capable of responding to the antigen is too small to incorporate detectable thymidine. Furthermore, it did not appear possible to enhance the response to homologous cells by previous immunization of the participating rabbits one with another. It is possible, however, that the procedure used for sensitization of the recipient was inadequate and it should be noted that the wide variation seen in the responses of unsensitized "mixes" makes it impossible to rule out the possibility that a small level of enhancement had occurred. There is, thus, a possibility that the response of cells in homologous mixes may be a secondary response to some antigenic grouping on the homologous cell which cross-reacts with an environmental antigen to which the rabbit has been previously exposed. This situation would be analogous to the ABO blood group system. It should be noted that cultures of human peripheral leukocytes do not respond to antigens such as tuberculin, etc., unless the patient is immune.

The rabbits used in this study were obtained from an outbred but closed stock and it is somewhat surprising that such a high frequency of positive responses (almost 100 per cent) should be obtained if it indeed represents a secondary response dependent on cross-reactions to environmental antigens. In each mix, however, the cells of either rabbit may respond to the antigenic de-

terminants in the other. It can be calculated that in a simple system involving only five antigenic types distributed equally in a population, 80 per cent of random mixes would give positive responses. A more complex system might, therefore, explain the frequency of responses observed.

Simonsen (41), Gowans (42), and others have studied the response of lymphoid cells injected into immunologically inert recipients. These responses have been taken as models of a primary homograft rejection. This conclusion should perhaps be reconsidered if it should be concluded that the present response is secondary rather than primary and that a comparison between the *in vivo* and *in vitro* systems is valid.

SUMMARY

An early proliferative response follows the mixing of homologous spleen or lymph node suspensions obtained from two unrelated rabbits. The rate of incorporation of radioactive thymidine has been used as a quantitative measure of this response. Thymus cells do not respond to homologous cell suspensions but may on occasion serve to stimulate the response in homologous spleen or lymph node cells. Homologous erythrocytes or autologous tissues do not stimulate a response.

No response occurs if the two cell populations are separated by a Millipore membrane.

Autoradiographic studies have established that 1 to 2 per cent of the intial cell population is involved in the response and they are large undifferentiated cells by the time they can first be identified as responders. There was no morphological evidence of any cellular interaction and the viability of mixed suspensions was not measurably different from that observed in separate suspension.

Simultaneous additive responses could be obtained to homologous cells and to antigen when cell suspensions from immunized rabbits were used. The interaction of the cell populations from the two rabbits did not appear to suppress the response of each to antigen.

The speed and magnitude of the response were in everyway comparable with the secondary response of cell suspensions from immunized rabbits exposed to the immunizing antigen. No evidence was obtained of any enhancement of the response to homologous cells by prior immunization with homologous tissues, but the possibility that it had occurred was not rigidly excluded.

The authors are indebted to Kay Colby and Gillian Page for technical assistance.

BIBLIOGRAPHY

1. Dutton, R. W., and Pearce, J. D., Antigen dependent stimulation of synthesis of deoxyribonucleic acid in spleen cells from immunized rabbits, *Nature*, 1962, **194**, 93.

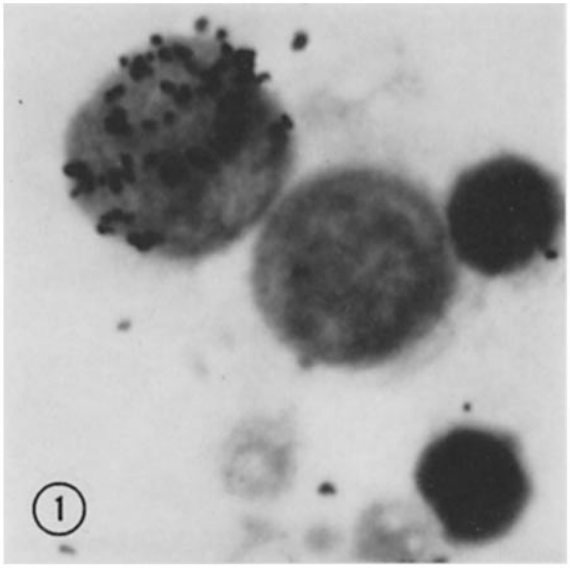
2. Dutton, R. W., and Pearce, J. D., An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response, *Immunology*, 1964, **7**, 40.
3. Chapman, N. D., Parkhouse, R. M. E., and Dutton, R. W., Antigen-stimulated proliferation in lymphoid and myeloid tissues from immunized rabbits, *Proc. Soc. Exp. Biol. and Med.*, in press.
4. Richardson, M., and Dutton, R. W., The appearance of antibody-synthesizing cells in spleen cell suspensions following secondary antigenic stimulation *in vitro*, *Science*, 1964, **146**, 655.
5. Dutton, R. W., and Bulman, H. N., The significance of the protein carrier in the stimulation of DNA synthesis by hapten protein conjugates in the secondary response, *Immunology*, 1964, **7**, 54.
6. Dutton, R. W., and Page, G., The response of spleen cells from immunized rabbits to cross-reacting antigens in an *in vitro* system, *Immunology*, 1964, in press.
7. Dutton, R. W., The effect of antigen on the proliferation of spleen cell suspensions from tolerant rabbits, *J. Immunol.*, 1964, in press.
8. Dutton, R. W., and Harris, G., Transfer of stimulation of deoxyribonucleic acid synthesis in antigen-stimulated rabbit spleen cell cultures, *Nature*, 1963, **197**, 608.
9. Dutton, R. W., and Parkhouse, R. M. E., Studies on the mechanism of antigenic stimulation in the secondary response, Proceedings of the Prague Symposium on Molecular and Cellular Basis of Antibody Formation, in press.
10. Bain, B., Vas, M., and Lowenstein, L., A reaction between leukocytes in mixed peripheral blood cultures, *Fed. Proc.*, 1963, **22**, 428.
11. Bain, B., Vas, M., and Lowenstein, L., The development of large immature mononuclear cells in mixed leukocyte cultures, *Blood*, 1964, **23**, 108.
12. Hirschhorn, K., Bach, F., Kolodny, R. L., Firschein, I. L., and Hashem, N., Immune response and mitosis of human peripheral blood lymphocytes *in vitro*, *Science*, 1963, **142**, 1185.
13. Bach, F., and Hirschhorn, K., Lymphocyte interaction: a potential histocompatibility test *in vitro*, *Science*, 1964, **143**, 813.
14. Rubin, A. L., Stenzel, K. H., Hirschhorn, K., and Bach, F., Histocompatibility and immunological competence in renal homotransplantation, *Science*, 1964, **143**, 815.
15. Hashem, N., and Carr, D. H., Mitogenic stimulation of peripheral lymphocyte cultures by autologous lymphocyte extracts in autoimmune diseases, *Lancet*, 1963, **2**, 1030.
16. Hashem, N., and Rosen, F. S., Mitogenic fraction in human peripheral lymphocyte extracts, *Lancet*, 1964, **1**, 201.
17. Pearmain, G., Lycette, R. R., and Fitsgerald, P. H., Tuberculin-induced mitosis in peripheral blood leucocytes, *Lancet*, 1963, **1**, 637.
18. Marshall, W. H., and Roberts, K. B., Tuberculin-induced mitosis in peripheral blood leukocytes, *Lancet*, 1963, **1**, 773.

19. Elves, M. W., Roath, S., and Israëls, M. C. G., The response of lymphocytes to antigen challenge *in vitro*, *Lancet*, 1963, **1**, 806.
20. Elves, M. W., Roath, S., Taylor, G., and Israëls, M. C. G., The *in vitro* production of antibody lymphocytes, *Lancet*, 1963, **1**, 1292.
21. Lycette, R. R., and Pearmain, G. C., Further observations on antigen-induced mitosis, *Lancet*, 1963, **2**, 386.
22. Cowling, D. C., Quaglino, D., and Davidson, E., Changes induced by tuberculin in leukocyte cultures, *Lancet*, 1963, **2**, 1091.
23. Ling, N. R., and Husband, E. M., Specific and nonspecific stimulation of peripheral lymphocyte, *Lancet*, 1964, **1**, 363.
24. Schuek, R., Cell transformation and mitosis produced *in vitro* by tuberculin purified protein derivative in human blood cells, *Am. Rev. Resp. Dis.*, 1963, **87**, 734.
25. Hirschhorn, K., Kolodny, R. L., Hashem, N., and Bach, F., Mitogenic action of phytohaemagglutinin, *Lancet*, 1963, **2**, 305.
26. Hashem, N., and Barr, M. L., Mitogenic effect of rabies vaccine on cultures of lymphocytes in disease of the nervous system, *Lancet*, 1963, **2**, 1029.
27. Hashem, N., Hirschhorn, K., Sedlis, E., and Holt, L. E., Infantile eczema evidence of autoimmunity to human skin, *Lancet*, 1963, **2**, 269.
28. Gräsbeck, R., Nordman, C., and De La Chapelle, A., Mitogenic action of antileucocyte immune serum on peripheral leucocytes *in vitro*, *Lancet* 1963, **2**, 385.
29. Gräsbeck, R., Nordman, C., and De La Chapelle, A., The leucocyte-mitogenic effect of serum from rabbits immunized with human leucocytes, Acta Med. Scand., 1964, suppl. **412**, 39.
30. Nowell, P. C., Phytohaemagglutinin: an initiator of mitosis in cultures of normal human leukocytes, *Cancer Research*, 1960, **20**, 462.
31. Elves, M. W., and Wilkinson, J. F., Effects of phytohaemagglutinin on the morphology of cultured leucocytes, *Nature*, 1962, **194**, 1257.
32. Elves, M. W., and Israëls, M. C. G., The mitogenic property of phytohaemagglutinin: studies on human leucocytes, *Brit. J. Haematol.*, 1963, **9**, 406.
33. Tanaka, Y., Epstein, L. B., Brecher, G., and Stohlman, F., Transformation of lymphocytes in cultures of human peripheral blood, *Blood*, 1963, **2**, 2614.
34. Epstein, L. B., and Stohlman, F., RNA synthesis in cultures of normal human peripheral blood, *Blood*, 1964, **24**, 69.
35. Nordman, C., De La Chapelle, A., and Gräsbeck, R., The interrelations of erythro-agglutinating, leuco-agglutinating and leucocyte-mitogenic activities in *Phaseolus vulgaris* phytohaemagglutinin, *Acta Med. Scand.*, 1964, suppl. **412**, 49.
36. Mackinney, A. A., Stohlman, F., and Brecher, G., The kinetics of cell proliferation in cultures of human peripheral blood, *Blood*, 1962, **19**, 349.
37. Cooper, E. H., Barkhan, P., and Hale, A. J., Observations on the proliferation of human leucocytes cultured with phytohaemagglutinin, *Brit. J. Haematol.*, 1963, **9**, 101.
38. Chapman, N. D., and Dutton, R. W., The *in vitro* stimulation of DNA synthesis in cultures of lymph node and spleen cell suspensions by homologous cells, *Fed. Proc.*, 1964, **23**, 353.

39. Parkhouse, R. M. E., and Dutton, R. W., Medium requirements and culture conditions for the antigen-dependent DNA synthesis of spleen cells *in vitro*, *Fed. Proc.*, 1964, **23**, 389.
40. Parkhouse, R. M. E., unpublished observations.
41. Simonsen, M., The impact on the developing embryo and newborn animal of adult homologous cells, *Acta Path. et Microbiol. Scand.*, 1957, **40**, 480.
42. Gowans, J. L., The fate of parental strain small lymphocytes in F_1 hybrid rats, *Ann. New York Acad. Sc.*, 1962, **99**, 432.

EXPLANATION OF PLATE 1

FIG. 1. The cells illustrated were obtained from a mixed suspension of lymph node cells from two unrelated rabbits. They were incubated for 19 hours and then given a 1 hour pulse of tritiated thymidine. They were then washed and reincubated in the presence of cold thymidine for a further 28 hours. Giemsa stain. Approximately X 2400,



(Chapman and Dutton: Stimulation of DNA synthesis)