*Immunology*, 1969, **16**, 381.

# The Relationship between Lymphocyte Transformation and Immune Response

## I. RATIO OF TRANSFORMING CELLS TO ANTIBODY FORMING CELLS

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(*Received 28th August* 1968)

**Summary.** A comparison was made between the number of cells forming or carrying antibody and those stimulated to transform *in vitro* by antigen, by using blood lymphocytes from rabbits immunized against sheep erythrocytes.

In blood samples collected 7–77 days after a single immunization, the number of transformed cells was always markedly higher than that of cells forming plaques or rosettes.

It is suggested that unsensitized cells are induced to transform by a stimulus transferred from other reacting cells.

## INTRODUCTION

Peripheral blood small lymphocytes from immunized animals may undergo a transformation reaction *in vitro* when cultured in the presence of the immunizing antigen. The reaction is characterized by increased metabolic activities, a morphological change and mitotic activity and resembles that induced by 'unspecific stimulants' like phytohaemagglutinin or by antisera against immunoglobulins or lymphocytes (Berman, 1966; Benezra, Gery and Davies, 1967; Dutton, 1967; Vischer and Stastny, 1967). Determination of the percentage of transformed cells has become a major method for assessing the extent of the response, although its quantitative relationship to the degree of sensitization at the cell level has not been determined.

The present study was carried out in order to compare in a population of lymphocytes from immunized animals the number of cells producing or carrying specific antibody to that of lymphocytes undergoing transformation when cultured with the antigen. This comparison could be made after finding that blood lymphocytes from rabbits immunized against sheep red blood cells (SRBC) are stimulated to transform *in vitro* by this antigen. Cells producing or carrying antibodies to SRBC can be enumerated by the use of formation of plaques in gel or rosettes in suspension.

## MATERIALS AND METHODS

### *Immunization*

Male or female mongrel rabbits weighing 3–4 kg were injected intravenously with 1·0 ml of a 15 per cent thrice washed sheep blood cell suspension.

*Cell suspensions*

Blood samples were collected at different intervals by cardiac puncture and the lymphocytes were partially separated as described elsewhere (Benezra *et al.*, 1967). These white cell suspensions consisted of 80–95 per cent small lymphocytes, contaminated with polymorphonuclear leucocytes and a few larger monocytes. After three washings with M-199 medium, the cells were tested in parallel for three activities, as follows:

(*a*) *Plaque formation in gel.* The technique of Jerne, Nordin and Henry (1963) was used for enumeration of plaque forming cells (PFC) of the 19S type. Later, the same agar plates were used for determination of the number of 7S type PFC: the complement was removed and goat anti-rabbit IgG was applied, as described by Wortis, Taylor and Dresser (1966).

(*b*) *Rosette formation (immunocyto-adherence test).* The technique of Zaalberg, van der Meul and van Twisk (1966) was used, with some modifications. The lymphocyte suspension ($2–4\times 10^6$) was gently rotated with $4\times 10^7$ SRBC in M-199 medium (1·0 ml final volume) for 30 minutes at 37° and then incubated for a further 18 hours at 4°. Rosettes were counted in blood counting chambers, in volume samples of at least 1·8 $mm^3$. Lymphocytes to which five or more sheep erythrocytes were attached were considered rosette-forming cells (RFC), but in most cases more erythrocytes were attached, forming small sphericals. RFC were identified by adding 1 per cent acetic acid into the chambers and in most cases were found to be small lymphocytes.

(*c*) *Transformation in culture.* Lymphocyte cultures, set up as described by Benezra *et al.* (1967) were stimulated by 0·1 ml of 2 per cent SRBC. The degree of response was determined after 5 days of incubation at 37° with 5 per cent $CO_2$ by both percentage of transformed lymphocytes and the uptake of tritiated thymidine ($^3$HT) (Benezra *et al.*, 1967). The radioactivity in the TCA—precipitate of the cells was determined in the present experiments after dissolving each precipitate with 0·5 ml formic acid and 3·0 ml ethanol. Seven millilitres of scintillation fluid (0·5 per cent PPO and 0·03 per cent POPOP in toluene) were then added to each sample, for counting in a Tricarb Packard scintillation spectrometer.

The viability of the lymphocytes in culture was determined by the trypan blue exclusion test (incubation with 0·2 per cent trypan blue for 10 minutes).

The proportion of lymphocytes entering mitosis every hour was assessed by harvesting cultures at different intervals after addition of colchicine (Amend Drug & Chemical Co. Inc. New York), at $5\times 10^{-7}$ M final concentration.

## RESULTS

Table 1 summarizes the activities performed by cells from rabbits at different intervals after immunization. The data presented in this paper were obtained with lymphocytes from rabbits with highly developed transformation activity, i.e. 7 days or more after immunization. The tested blood samples contained small numbers of plaque forming cells (PFC) of the 19S type, and more PFC were detected when those of the 7S type were also exposed. The numbers of rosette-forming cells (RFC) were greater than those of PFC by a factor of more than 100, a difference which is in accord with the findings of Zaalberg *et al.* (1966) with mouse spleen cells. A marked transformation reaction was found in all tested rabbits, as shown by both the increased $^3$HT uptake and values of cell transformation. The numbers of transformed cells exceeded in all cases those of cells

TABLE 1

COMPARISON BETWEEN TRANSFORMATION AND ANTIBODY FORMATION BY LYMPHOCYTES FROM RABBITS IMMUNIZED AGAINST SRBC

| Rabbit No. | Days after immunization | Plaques/$10^3$ cells | | Rosettes/$10^3$ cells | Transformed cells*/$10^3$ cells experimental/control | $^3$HT uptake (counts/min) experimental/control |
|---|---|---|---|---|---|---|
| | | 19S | 19S+7S | | | |
| C-60 | 7 | 0·001 | ND† | 0·325 | 91/4 | 1,678/70 |
| C-23 | 8 | 0·023 | 0·055 | 0·230 | 159/46 | 24,742/1,142 |
| C-32 | 9 | 0·005 | ND | 0·433 | 266/61 | 8,350/296 |
| C-51 | 15 | 0·002 | 0·044 | 1·880 | 189/33 | 33,922/1,435 |
| C-53 | 15 | 0·003 | 0·073 | 3·700 | 115/5 | 4,943/90 |
| C-27 | 19 | 0·009 | ND | 1·250 | 110/15 | 1,102/82 |
| C-13 | 36 | 0·001 | ND | 0·550 | 127/28 | 828/102 |
| C-51 | 77 | <0·001 | <0·001 | 0·753 | 61/22 | 2,296/550 |

* Including blast cells and mitotic figures. Frequency of mitotic figures ranged from 1 to 5/$10^3$ in experimental cultures and was zero in all controls except that of C-32 which gave 1/$10^3$.

† Not done.

carrying or forming antibody, the maximal difference being found in rabbit C-32, with 473 transformed cells (net) as against one RFC or 41,000 as against one PFC (of the 19S type).

The rates of death and proliferation in the lymphocyte cultures were determined in order to estimate the effect of these factors on increasing the proportion of the transformed cells. White cell counts and trypan blue exclusion tests showed that death and disintegration of cells ranged from 50 to 80 per cent of the original suspensions on the 5th day of culture.

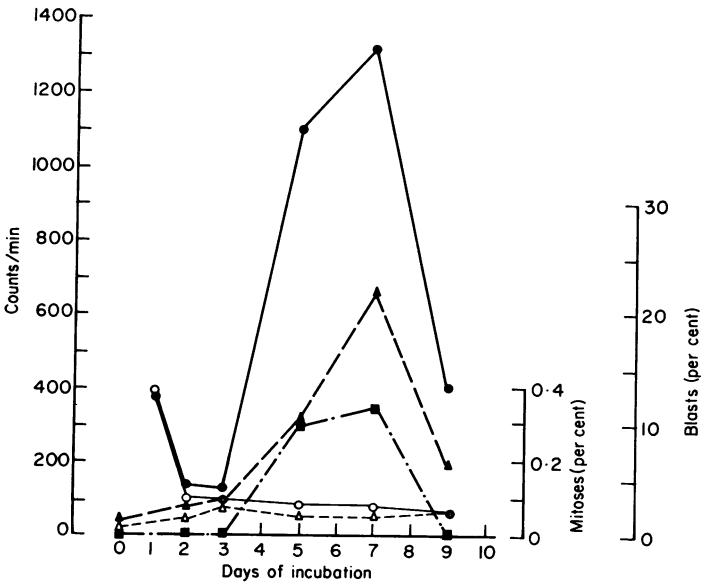


FIG. 1. Kinetics of responses of lymphocytes from rabbit C-27, collected 19 days after immunization with SRBC. Experimental cultures were stimulated with 0·1 ml of 2 per cent SRBC, no stimulants being added to the controls. Percentages of blast cells and mitoses were determined in cultures harvested on the corresponding days. Uptake of $^3$HT (counts/min) was determined after a pulse of 24 hours; the time of harvesting being recorded in this figure. ●, Counts/min; ○, counts/min (control); ▲, blasts; △, blasts (control); ■, mitoses.

Cell death, however, was shown by Chalmers, Cooper, Evans and Topping (1967) to affect both small and transformed lymphocytes and the corrected proportion of blastoid cells should not, therefore, deviate considerably from that found on harvesting. The proliferation of transformed cells was estimated to increase the number of blastoid cells by a factor of less than 8. This estimate was based on the finding that increased uptake of $^3$HT followed by mitotic activity, began on the 3rd or 4th day of culture (Fig. 1), i.e. 24–48 hours before harvesting. The generation time of these cells is assumed to resemble that of other lymphocytes in culture (see Baker and Landy, 1967), i.e. in the range of 12–20 hours (Sasaki and Norman, 1966; Johnson, Chan, Lobue, Monette and Gordon, 1967). Thus, not more than three generations can occur in these cultures, with only a small portion of the cells completing more than two divisions. The proportion of lymphocytes entering mitosis every hour on the 4th or 5th day of culture was determined in cultures of

TABLE 2

FORMATION OF MITOSES IN CULTURES OF LYMPHOCYTES FROM IMMUNIZED RABBITS

| Rabbit No. | Days after immunization | Days in culture | Transformed cells (per cent) | Mitotic index (per cent)* | New mitosis/hour (per cent)* |
|---|---|---|---|---|---|
| C-60 | 7 | 5 | 9·1 | 0·13 (1·42) | 0·13 (1·42) |
| C-51 | 14 | 5 | 18·9 | 0·50 (2·64) | 0·50 (2·64) |
| C-53 | 14 | 4 | 3·0 | 0·13 (4·33) | 0·08 (2·66) |
| C-51 | 77 | 5 | 6·1 | 0·11 (1·80) | 0·08 (1·31) |
| C-32 | 5† | 5 | 20·9 | 0·32 (1·53) | 0·38 (1·81) |

* Percentage based on total lymphocytes; in parentheses, percentage based on transformed cells.

† After a booster immunization.

some rabbits by the addition of colchicine. The values were found to be similar to the mitotic indices of the corresponding cultures, i.e. 0·08–0·50 per cent of the total population or 1·31–2·66 per cent of the transformed cells (Table 2). The latter values are similar to those found in cultures of human lymphocytes stimulated by phytohaemagglutinin (Astaldi and Airo, 1967) and this may support the assumption that the generation times of the two cell populations are not markedly dissimilar.

## DISCUSSION

The blast formation and cell proliferation that follow the reaction between lymphocytes from immunized animals and the specific antigen are assumed to represent a memory response (Dutton, 1967; Vischer and Stastny, 1967). This is in accord with data showing that circulating small lymphocytes are carriers of immunological memory (Gowans and Uhr, 1966). It has been suggested that the transformation response is triggered by the reaction between the antigen and specific antibodies ('receptors'), located on the cell surface (see Dutton, 1967). Thus, if one assumes that all blasts are transformed sensitized cells, one may expect a similar number of antibody carrying and transforming cells in culture. Rosette-forming cells (RFC) represent in this study a population of antibody carrying cells and indeed these cells have been suggested by Zaalberg, van der Meul and van Twisk, (1968) to be involved in the memory apparatus of the animal.

The results presented in this study show that in immunized rabbits with fully developed activity of lymphocyte transformation, the number of transformed cells is larger than that

of cells producing plaques or carrying (RFC) antibody. This difference is only partially due to cell proliferation and other reasons must be considered for its explanation. First, the transformation response may be a highly sensitive test, that activates cells carrying antibody in quantities undetected by other tests. Alternatively, most blastoid cells were 'normal' lymphocytes that were induced to transform by a transferred stimulus, originating in a few sensitized (antibody carrying) cells. The third explanation suggests that the antigen-sensitive cells in the culture are devoid of conventional antibody to SRBC and can be stimulated by antigen 'processed' in other cells.

The first hypothesis is in line with the assumption of Sell (1967) that the high transformation percentages (up to 80 per cent) induced in rabbit lymphocytes by antiserum to a single immunoglobulin are due merely to the sensitivity of the technique. This assumption, that all transforming cells carry the specific globulin, leads to the unexpected conclusion, that about 25 per cent of the lymphocytes produce all three immunoglobulins. The analogous hypothesis, that all cells reacting to antigen carry antibody, does not fit well with the finding that the reaction to a specific antigen involves high proportions of the circulating lymphocytes. Moreover, lymphocytes from donors immunized against two different antigens do not usually show a summation effect when cultured with both antigens (Bouveng, Gardell, Low and Norden, 1967; Caron, 1967; Gery, Benezra and Davies, to be published). As a lymphoid cell reacts usually to a single antigen only (see Green, Vassalli, Nussenzweig and Benacerraf, 1967), these data rather support the alternative hypothesis, that a transfer process takes place in the reaction; the lack of summation may be attributed to competition between the two independent responses for cells or materials that participate in the transfer reaction and serve to limit its extent. The concept of a transfer process among cultured lymphocytes is strongly supported by indirect evidence (Mayron and Baram, 1967; Moorhead, Connolly and McFarland, 1967), as well as by preliminary direct evidence (Bloom and Bennett, 1968).

Morphological studies of lymphocyte cultures show actual interaction between cells in cultures (McFarland and Heilman, 1965; Berman, 1966) and suggest, too, that a transfer process takes place in culture. Most interactions *in vitro* involve macrophages and lymphocytes and the apparent activity of the phagocytes may support the third mentioned hypothesis, namely, that antigen-sensitive cells are stimulated by processed antigen and do not carry conventional antibodies.

Blood samples collected 4–6 days after immunization with SRBC were found to contain great numbers of PFC (up to $396/10^6$) along with minimal or no transformation activity (data to be published). These findings indicate that the two cell populations are formed separately, with the peak of antibody-forming cells preceding that of 'memory' cells.

## ACKNOWLEDGMENTS

This study was supported by a grant from the Israel Cancer Association and Grant HE-05739 from the National Institutes of Health, United States Department of HEW.

We thank Mrs Ora Eyal for her assistance.

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