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The Immunological Significance in the Guinea-Pig of *In vitro* Transformation of Lymph Node, Spleen and Peripheral Blood Lymphocytes

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Summary. Cells from lymph nodes or spleen, or peripheral blood leucocytes of immunized guinea-pigs were cultured in the presence of antigens or phytohaemagglutinin. Significant incorporation of tritiated thymidine occurred in a variable proportion of the experiments with lymphocytes from each of the three sources. Cells taken from animals that had been immunized with sheep erythrocytes with adjuvant, and which showed strong delayed hypersensitivity, and from animals immunized intravenously with sheep erythrocytes, which failed to show delayed hypersensitivity reactions, both responded to sheep erythrocytes *in vitro*.

Cells from guinea-pigs immunized with complete Freund's adjuvant alone, which showed strong delayed hypersensitivity to tuberculin PPD, gave more positive responses *in vitro* than did cells taken from animals which received an intravenous injection of tuberculin PPD before the adjuvant. These animals showed no or weak delayed hypersensitivity reactions (immune deviation).

The immunological significance of the *in vitro* proliferative reaction is discussed.

INTRODUCTION

In vitro lymphocyte transformation of human peripheral blood cells (Nowell, 1960) and of splenic cells of the rabbit (Dutton and Eady, 1964) has been extensively studied. The significance of this event, however, in terms of *in vivo* immunological events was difficult to interpret from the results of such studies. Work with guinea-pig cells is more promising in this respect, since both a state of delayed hypersensitivity and production of circulating antibody can readily be induced, and in certain circumstances one of these phenomena can be studied in relative isolation from the other. Studies along such lines by Oppenheimer, Wolstencroft and Gell (1967) and by Mills (1966) have strongly suggested the *in vitro* reaction to be positively correlated with the donor's state of delayed hypersensitivity rather than with circulating antibody production. Such observations prompted us to test the reactions of guinea-pigs that showed immune deviation, a phenomenon recently explored by Asherson and Stone (1965) and by Loewi, Holborow and Temple (1966). At the same time we have compared the effect of intravenous immunization and immunization with adjuvant on the *in vitro* response to sheep erythrocytes,

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thus attempting to make correlations between the *in vitro* lymphocyte response and the *in vivo* immune response of the guinea-pig to a particulate antigen.

MATERIALS AND METHODS

Immunization

Sheep blood was obtained from Burroughs Wellcome. The cells were washed three times and a dose of 4×10^9 given to guinea-pigs either intravenously or with complete Freund's adjuvant into the rear footpads. For 'immune deviation' 1 mg PPD (obtained by courtesy of the Ministry of Agriculture, Fisheries and Food, at Weybridge) was injected intravenously in the form of an alum preparation, followed 1–2 weeks later by 0·4 ml complete Freund's adjuvant in the rear footpads. Other guinea-pigs received only Freund's adjuvant. Twenty-four hours before cell harvest, guinea-pigs were skin tested with 10 μg PPD, or with 0·1 ml of a 5 per cent sheep cell suspension, or both.

Cells

Two weeks after immunization (counted in deviation experiments, from the date of immunization with adjuvant), the guinea-pigs were bled by cardiac puncture. The heparinized blood (10 μg heparin/ml blood) was sedimented after mixing with 50–100 per cent of its volume of 3 per cent gelatin. After standing at 37° for approximately 1 hour, the plasma was removed and the centrifuged cells were washed twice in tissue culture medium. Total and differential counts were made.

Lymph nodes were dissected from the popliteal, inguinal and flank regions of the guinea-pigs. From animals immunized intravenously, cervical and mesenteric nodes were also taken. Spleens were removed at the same time. These tissues were rinsed in Parker's tissue culture medium 199 (Glaxo). Nodes and spleens were finely minced with scissors and the particles crushed repeatedly with Spencer Wells' clamps. The cell suspension was then pipetted away from the sediment of connective tissue. Cells were centrifuged in medium 199 with 5 per cent foetal calf serum. They were re-suspended in a small volume of NCTC 109 (Difco, Detroit) and counted. The great majority of cells in the spleen and lymph node preparations appeared to be small and large lymphocytes. The preparations from peripheral blood varied between 75 and 95 per cent small lymphocytes, most of the remainder being polymorphs. The viability of the cells was not assessed.

Culture

Cell concentrations were adjusted to 10^7 cells per tube containing a volume of 2·5 ml. For peripheral blood lymphocytes, the final concentration was $1{\cdot}25–1{\cdot}5 \times 10^6$/2·5 ml. The vessels were Falcon brand plastic tubes. The culture medium consisted of 60 per cent NCTC 109 (Difco) and 40 per cent heat-inactivated foetal calf serum, 2000 units penicillin per 100 ml and 9 mg streptomycin per 100 ml.

Antigens were added when the cultures were set up, as follows: PPD 1 μg, or 10 μg/ml; sheep erythrocytes, 10^8 or 10^9 cells/2·5 ml culture; 10, 100, 200 μg or 1 mg BGG/ml. As a non-specific stimulant, phytohaemagglutinin M (Difco) was added, 0·1 ml/2·5 ml culture. Each culture was usually set up in triplicate.

The tubes were put in a desiccator and gassed with 5 per cent CO_2–air. Incubation was at 37°. After 1 day of incubation, 2·5 μc tritiated thymidine (Radiochemical Centre, Amersham) were added to phytohaemagglutinin cultures which were subsequently harvested on the 3rd day. For all other cultures, the culture medium was partially renewed

on the 2nd or 3rd day, about 60 per cent of the medium being replaced, leaving the cells undisturbed at the bottom of the tube. On the 3rd and 4th days these cultures received tritiated thymidine. On the 5th day, all cultures, except those with phytohaemagglutinin, were harvested, At this stage, tritium was added to a few tubes which had not previously received this, and the culture terminated immediately. These tubes served as a check on the completeness of the washing procedure for the removal of non-DNA-incorporated tritium.

The culture cells were centrifuged at 150 *g*, re-suspended in saline and frozen and thawed for cell disruption. Where erythrocytes had been added as antigen, rapid lysis in water, followed by re-suspension in saline was carried out. After cell disruption cold trichloro-acetic acid was added to a final concentration of 0·3 M. The tubes were centrifuged for 10 minutes at approximately 1000 *g* in the cold. The supernatant was removed, and the precipitate dispersed in dimethyl-sulphoxide (44 per cent solution in buffered saline). After another centrifugation at approximately 2000 *g* for 30 minutes, the resultant precipitate was taken up in absolute ethanol and re-centrifuged. Finally, the precipitate was dissolved in 1 ml 0·1 N NaOH and this was added to 15 ml Bray's mixture with Cab-O-Sil (Packard) for counting in a scintillation counter. This method is described in detail by Vischer and Stastny (1967).

RESULTS

Table 1 shows the results of cultures of cells from lymph nodes, spleens, and peripheral white cells of immunized guinea-pigs. When the counts of cultures with antigen were two or more times the counts of controls without antigen, the result was considered to be positive. Counts are shown in detail in Figs. 1 and 2. Table 1 includes all results from animals tested at any time after immunization, while Figs. 1 and 2 only refer to animals immunized for our standard period of 2 weeks. We obtained significant responses to PPD and also to sheep erythrocytes as antigens. Mills (1966) and Oppenheim *et al.* (1967) have previously reported successful antigen-dependent stimulation of guinea-pig lymphocytes in culture. We obtained rather more successful results with lymph node cells

TABLE 1

RESULTS OF TESTING LYMPHOCYTES WITH IMMUNIZING ANTIGEN

Source of lymphocyte	Lymph node	Spleen	Peripheral white blood cells
Immunized complete Freund's adjuvant tested PPD	8/12	9/13	7/9
Immunized sheep RBC + adjuvant tested RBC	6/8	4/7	2/7
Immunized RBC i.v. tested RBC	4/4	2/5	3/6

Numerator is number of experiments, each experiment referring to one animal, in which counts in test were twice or more the number of counts of no-antigen control. Denominator is the total number of animals tested for each antigen, for cells from each of the three sources. Amount of antigen is PPD 1 or 10 μg/ml; sheep erythrocytes 10^8 or 10^9/culture of 2·5 ml.

than with cells derived from either spleen or peripheral blood, but this distinction was not as marked as in the work of Oppenheim *et al.* (1967). We have also obtained some positive results using bovine γ-globulin as antigen with cells from guinea-pigs immunized with this antigen either with Freund's adjuvant intracutaneously or without adjuvant intravenously. Although controls with cells from unimmunized animals were usually negative, we have occasionally obtained, as did Oppenheim *et al.* (1967) increased

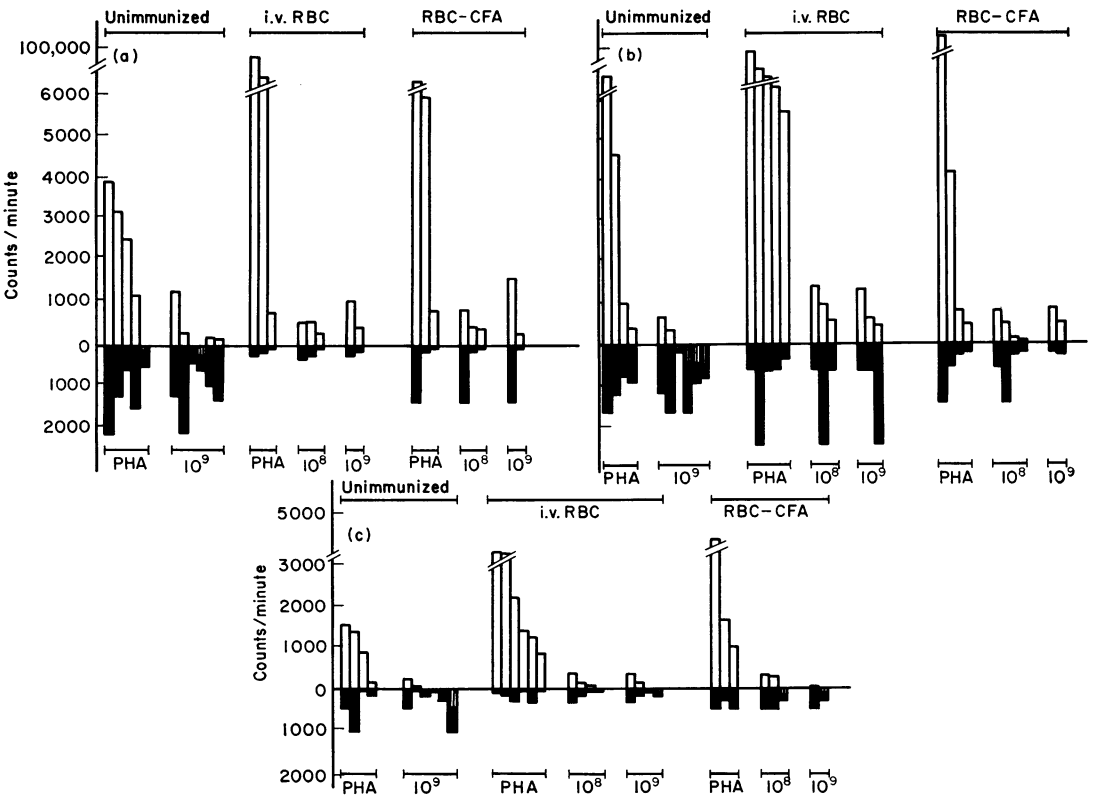

FIG. 1. Tritiated thymidine incorporation by cultured lymphocytes of unimmunized guinea-pigs, guinea-pigs immunized intravenously with sheep erythrocytes, and guinea-pigs immunized intracutaneously with sheep cells and complete adjuvant. The response of 10^8 and 10^9 sheep cells per culture is recorded, as well as to phytohaemagglutinin. Black column = unstimulated culture; white column = increment of stimulated over unstimulated; hatched column = decrement of stimulated cultures. In some cultures, figures for only one of the concentrations of erythrocytes are available, in others for both. (a) Shows results of lymph node cells; (b) of spleen cells; and (c) of peripheral blood lymphocytes.

incorporation of tritiated thymidine by cells from such animals, when exposed to PPD, and also rarely to sheep erythrocytes as shown in Fig. 1. Phytohaemagglutinin was usually used as a non-specific stimulant in these experiments as a demonstration of the viability of the culture.

In our experiments with sheep erythrocytes, significantly increased tritiated thymidine incorporation was obtained with lymph node cells, from animals either immunized intravenously or with adjuvant intracutaneous. Spleen and particularly blood lymphocytes

provided rather fewer positive results but here also the route of immunization did not appear to be the governing factor. Cutaneous tests for delayed hypersensitivity to sheep erythrocytes in intravenously immunized animals gave negative or very weak reactions, whereas all guinea-pigs that had been immunized with sheep cells and adjuvant had strongly positive skin tests at 24–48 hours. Titres of agglutinating and lytic antibodies to sheep cells were similar in the two groups. The experiments with sheep erythrocytes thus

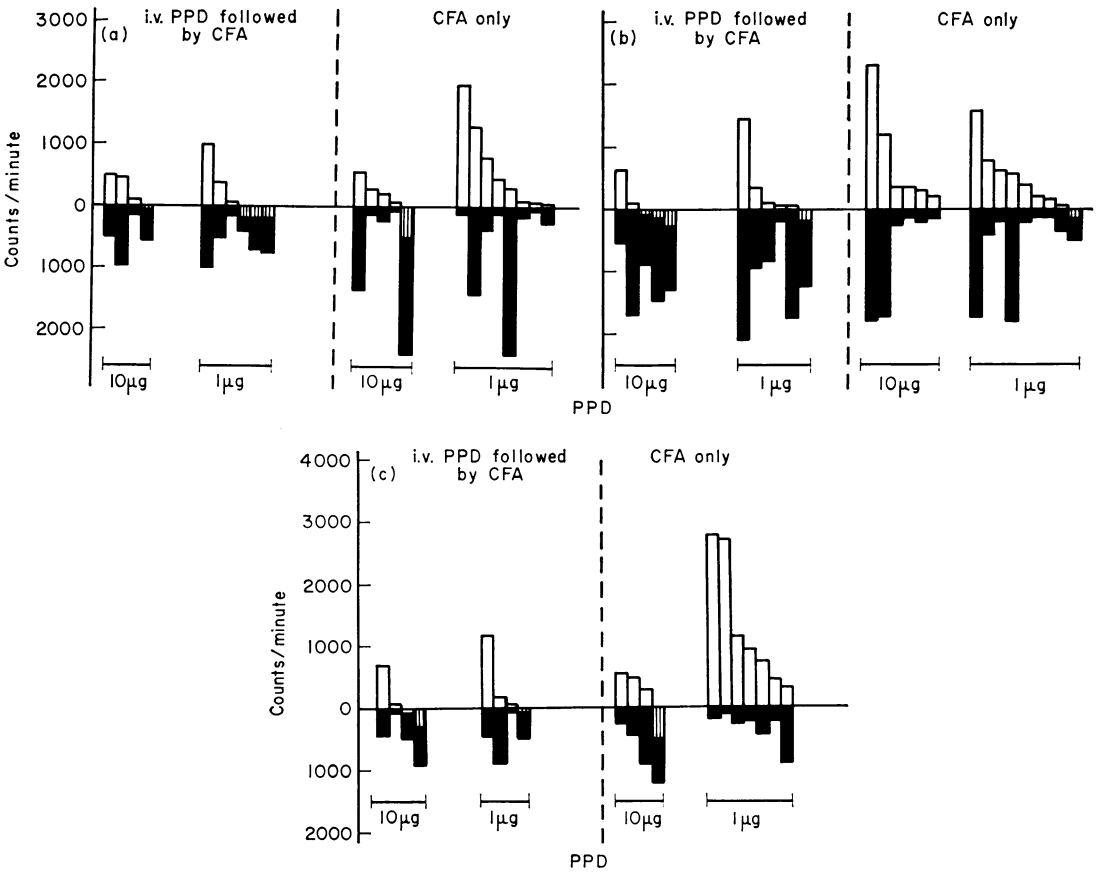

FIG. 2. Tritiated thymidine incorporation by cultured lymphocytes of guinea-pigs immunized with intravenous PPD followed by intracutaneous complete adjuvant, and complete adjuvant only. Cells obtained from lymph nodes (a), spleen (b), and blood (c). Responses to 10 and 1 μg PPD/ml are given, but both were not available in every experiment. Black column = unstimulated culture; white column = increment of stimulated over unstimulated; hatched column = decrement of stimulated cultures.

did not show the relationship to route of immunization and delayed hypersensitivity that was reported by Mills (1966) using ovalbumin.

Fig. 2 shows results obtained with lymphocytes from guinea-pigs that had been immunized with complete Freund's adjuvant, as opposed to another group similarly immunized, but after a preceding intravenous injection of PPD. This system of immune deviation was explored by Asherson and Stone (1965). The former group of animals,

when tested before the cell harvest 2 weeks after adjuvant immunization had delayed hypersensitivity reactions to PPD averaging 20 mm in diameter and extensively indurated, while the latter group showed considerably reduced reactions of around 6–8 mm with little induration. The experiments were conducted to show whether this immunological defect was associated with an impaired tritiated thymidine uptake of lymphocytes, from the inhibited animals, during stimulation *in vitro*. Results in Fig. 2 suggest that this is so, although there are not enough results for statistical treatment and the scatter is very wide. The trend of results is similar for lymphocytes from lymph nodes, spleen and blood. An antigen concentration of 1 μg PPD/ml seemed to be more successful than 10 μg/ml in most of these experiments.

DISCUSSION

Although successful lymphocyte culture is not as readily achieved in the guinea-pig as it is in man (Nowell, 1960; Pearmain, Lycette and Fitzgerald, 1963) or in the rabbit (Dutton and Eady, 1964) such experiments are of particular value, since most of the information on delayed hypersensitivity has been obtained in this species. In many instances, the immune responses of circulating antibody formation and of delayed hypersensitivity can be differentiated, thus offering a basis of assessment of the possible meaning of lymphocyte proliferation *in vitro* in relation to such responses. Our desire to investigate the status of lymphocytes in immune deviation meant that guinea-pigs had to be used in these experiments, since both we and other workers have investigated this phenomenon in this species.

With tuberculin PPD we obtained positive responses with cells from lymph nodes, spleens and peripheral white cells of adjuvant-immunized animals. Oppenheim *et al.* (1967) obtained responses in all their lymph node cell cultures, while their cultures of spleen and blood lymphocytes appear to have been somewhat less frequently successful with this antigen than were ours. The interval between immunization and testing which in our experiments was 2 weeks or more, and in those of Oppenheim *et al.* (1967) was 10 days, may partly account for this difference. It may be, as these authors suggest, that a re-distribution of the immune cell population from the lymph nodes draining the area of immunization namely the footpads, to spleen and blood, is a fairly late event. With lymph node cells, we did not obtain the uniformly low base-line of tritium incorporation found by Oppenheim *et al.* (1967) and this militated against our results being as successful as theirs in this instance. This difference may be due to their addition of tritiated thymidine only for the last few hours of incubation, whereas we have added this at an earlier stage. Chapman, Parkhouse and Dutton (1964), whose rabbits were immunized intravenously, obtained evidence of antigen-induced cell proliferation *in vitro* with spleen and lymph node cells, but not with peripheral blood, bone marrow, thymus or pulmonary alveolar macrophages.

As already mentioned, results with 1 μg PPD were frequently better than those obtained with 10 μg. With sheep erythrocytes, a concentration of 10^7 was uniformly ineffective, while no quantitatively related response was apparent when concentrations of 10^8 and 10^9 cells per culture were compared. In this study we have relied, as have Dutton and Eady (1964), on the counts of DNA-incorporated tritium to assess the response of cultured cells, and have only studied the accompanying cell transformation in a small number of

autoradiographs. Oppenheim *et al.* (1967) however, consistently observed labelled cells and total counts to provide results in agreement with one another.

There is much evidence to suggest a parallelism between the state of delayed hypersensitivity in the guinea-pig and the response of lymphocytes from an immunized animal to antigen *in vitro*. The response is carrier-specific, it can take place when no antibody has been demonstrated in the lymphocyte donor with antigens such as guinea-pig albumin-orthanilic acid (Oppenheim *et al.*, 1967) or tuberculin PPD (although in two of our guinea-pigs used in these experiments we found a positive passive cutaneous anaphylaxis reaction with PPD). Our experiments with cells from animals which showed immune deviation to PPD further serve to support the concept of a relationship to delayed hypersensitivity, for the animals with diminished delayed hypersensitivity reactions showed fewer responses when their lymphocytes were tested with antigen *in vitro*. That the defect in immune-deviated animals is a cellular one was similarly indicated by the work of Asherson (1966) employing transfer of delayed hypersensitivity from deviated and control guinea-pigs. It is necessary to remember, however, that in immune deviation in animals immunized with a protein antigen such as ovalbumin, there is a lower level of serum γ_2 antibody than in undeviated animals which have received the antigen with complete adjuvant only (Loewi *et al.*, 1966). This constitutes evidence for a general effect on the immune response, and it is possible that it is this which is reflected in the *in vitro* lymphocyte proliferation response rather than the state of delayed hypersensitivity of the cell donor. The particular difficulty of investigating this point with PPD is the absence of readily demonstrable antibody; in the sheep cell system we obtained a proliferation response after intravenous immunization, and were therefore in no position to investigate the effect of immune deviation, which is readily produced with this antigen (Loewi and Temple, unpublished).

Our results with sheep cell immunization by the intravenous route and other unpublished experiments with intravenous bovine γ-globulin showed that such animals have a population of lymphocytes which is capable of responding to the immunizing antigen *in vitro*, although they showed no or only a slight degree of cutaneous delayed response. This is in agreement with the work of Dutton who has used a variety of antigens in rabbits by the intravenous route. On the other hand, Mills (1966) has recorded negative responses by the cells of guinea-pigs that had received ovalbumin intravenously, while the same antigen given intracutaneously with adjuvant appears to have produced a positive response.

We may conclude from the work of others and from our experiments reported here that an *in vitro* lymphocyte proliferation in response to antigen is shown by those immunized animals that show delayed hypersensitivity and these two events are positively correlated. We also consider, however, that this phenomenon may be shown by the lymphocytes of guinea-pigs that have been immunized without adjuvant and do not show a demonstrable degree of delayed hypersensitivity. The actual status of the lymphocytes undergoing proliferation *in vitro* with regard to any commitment towards antibody formation or delayed hypersensitivity potential remains unknown.

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