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# Induction of plaque-forming cells in human blood lymphocytes cultured in the presence of antigen and Epstein–Barr virus: a study with normal donors and infectious mononucleosis patients

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SUMMARY

Normal human peripheral blood lymphocytes, stimulated *in vitro* with SRBC in the presence of Epstein–Barr virus (EBV), gave rise to plaque-forming cells (PFC) specific for the antigen. PFC levels were very low before day 4 and increased thereafter, reaching a maximum around day 8. However, the kinetics of the response varied considerably from donor to donor and from experiment to experiment. In some instances a second peak of PFC was obtained beyond day 10. Large differences in the magnitude of the response were observed among different normal donors, the overall responsiveness range covering four orders of magnitude.

Peripheral blood lymphocytes from infectious mononucleosis patients in the acute stage of the disease, when a high titre of heterophil and anti-EBV antibodies were present, did not give rise to PFC. A return to normal responses was observed during recovery from the disease.

## INTRODUCTION

The Epstein–Barr herpesvirus (EBV) binds selectively to human B lymphocytes (Jondal & Klein, 1973; Greaves, Brown & Rickinson, 1975). Infection of human leucocytes with EBV *in vitro* induces DNA synthesis (Gerber & Hoyer, 1971), a polyclonal Ig production (Rosén *et al.*, 1977) and a lymphoblastoid transformation (Henle *et al.*, 1967; Pope, Horne & Scott, 1969; Gerber, Whang-Peng & Monroe, 1969; Miller *et al.*, 1971). We have shown that co-cultivation of normal human peripheral blood lymphocytes (PBL) with EBV and antigen results in the induction of a specific antibody response. This response depends on the presence of both stimuli (EBV and antigen) and is antigen specific (Luzzati, Hengartner & Schreier, 1977).

Several lines of evidence support the role of the EBV as a causative agent in the infectious mononucleosis (IM) (Henle & Henle, 1972). This disease is characterized by an intense lymphocyte proliferation, an increase of immunoglobulin levels (mainly IgM) and the development of heterophil antibodies, i.e. agglutinins directed against sheep erythrocytes (Carter, 1975). Recent progress in the research on EBV now permits a better understanding of the way in which this virus induces the typical clinical and immunopathological picture of the disease (Epstein & Achong, 1977a, b).

We felt it to be of interest to investigate the capacity of human PBL, already infected *in vivo* with EBV, to mount an antibody response *in vitro* to sheep red blood cells (SRBC) in the system described previously. For this purpose, we repeatedly bled IM patients at different stages of the disease and cultured their lymphocytes with SRBC, with and without further addition of fresh EBV.

In this paper we present the results obtained with peripheral blood lymphocytes from four patients and from thirty-six normal donors.

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## MATERIALS AND METHODS

*Virus preparation.* Virus stocks were prepared from supernatants of the EBV transformed marmoset cell line B95/8 (Miller & Lipman, 1973) ). The virus was pelleted by centrifugation at 100,000 *g* for 1 hr. The upper 90% or 95% of the supernatant was discarded and the pellet was resuspended in the remaining 10% or 5% or the volume (10× or 20× stocks).

Every concentrated stock of virus was tested with one or more blood donors. The preparations which induced a definitely lower response than a reference batch were discarded. The preparations were divided in aliquots and stored at 4°C. The material could be kept for several months without any loss of activity.

*Blood donors.* The majority of the normal donors were healthy employees of the Hoffmann-La Roche Company. The infectious mononucleosis patients were referred to us by local physicians. They were bled as soon as possible after the appearance of the first symptoms of the disease and then again one or more times at different intervals. In every instance haematological and serological tests were performed to ascertain the state of the illness.

*Cell preparation.* Heparinized blood obtained from healthy donors or from infectious mononucleosis patients was centrifuged on a Ficoll-Urovison gradient (Böyum, 1968). The plasma on top of the gradient and the cells at the interface were harvested. The cells were washed in $Ca^{++}Mg^{++}$ free Dulbecco's balanced salt solution and resuspended in autologous plasma (2·0 ml of plasma every 10 ml of starting blood). 6·0 ml of cell suspension were pipetted into a 20 ml syringe packed with 1·2 g of nylon wool (Coop, Basel, Switzerland, No. 609.002). With the aid of the syringe plunger the fluid was forced into the wool. After 45 min of incubation at 37°C, the cell suspension was squeezed out of the syringe and the wool was washed twice with 3·0 ml of warm Hanks' balanced salt solution (HBSS). The cells were then washed twice with HBSS.

*Tissue cultures.* The cells were resuspended at a density of $9 \times 10^6$/ml in a modification of the tissue culture medium described previously (Luzzati *et al.*, 1976) in which foetal calf serum (FCS) was substituted with human AB serum. This serum was heat-inactivated and absorbed three times in the cold with SRBC. 50 µl per ml of 1% SRBC were added as antigen. The cell suspension was distributed in aliquots of 0·1 ml in the wells of Microtest plates (Falcon Plastics, Oxnard, California No. 3040). 25–50 µl of concentrated virus preparation were then added to each each well. The volume required varied with different virus batches and was ascertained by pre-testing each new stock.

The plates were incubated at 37°C in an atmosphere of 5% $CO_2$. After 24 hr, 50 µl of nutritional cocktail, made as previously described (Luzzati *et al.*, 1976), but containing 10% absorbed human AB serum instead of FCS, were added to each well. Controls with virus alone and with antigen alone were included.

*Evaluation of the antibody response in vitro.* At the time of assay the contents of several wells (usually five) were pooled. The cells were washed, resuspended in Eagle's minimal essential medium (MEM) and tested for the presence of PFC by a modification of the method described previously (Luzzati *et al.*, 1976).

## RESULTS

### *Effect of nylon wool treatment*

The importance of the nylon wool filtration step was investigated. The results in Table 1 show that if the nylon treatment was omitted from the lymphocyte preparation procedure, no response could be obtained, although the capacity of the cells to survive in culture was not significantly modified.

Passage through nylon wool did not alter the proportion of cells capable of making rosettes with SRBC (T cells), nor the proportion of Ig-bearing cells (B cells) (unpublished results).

### *Kinetics of the response*

Only rarely could PFC be detected before day 4 and maximum response was mostly observed between days 6 and 9. However, the time course of the appearance of PFC varied considerably from donor to donor and from experiment to experiment. While in some cases PFC were present over 2 or 3 days only, in other

TABLE 1. Effect of nylon wool treatment

| Nylon | Day 7 cells* | Day 7 PFC† | Day 9 Cells | Day 9 PFC |
|---|---|---|---|---|
| No | 4·8 | 0 | 6·3 | 0 |
| Yes | 8·3 | 450 | 5·6 | 354 |

* Cells per well $\times 10^{-5}$ (starting number: $9 \times 10^5$).

† PFC per well.

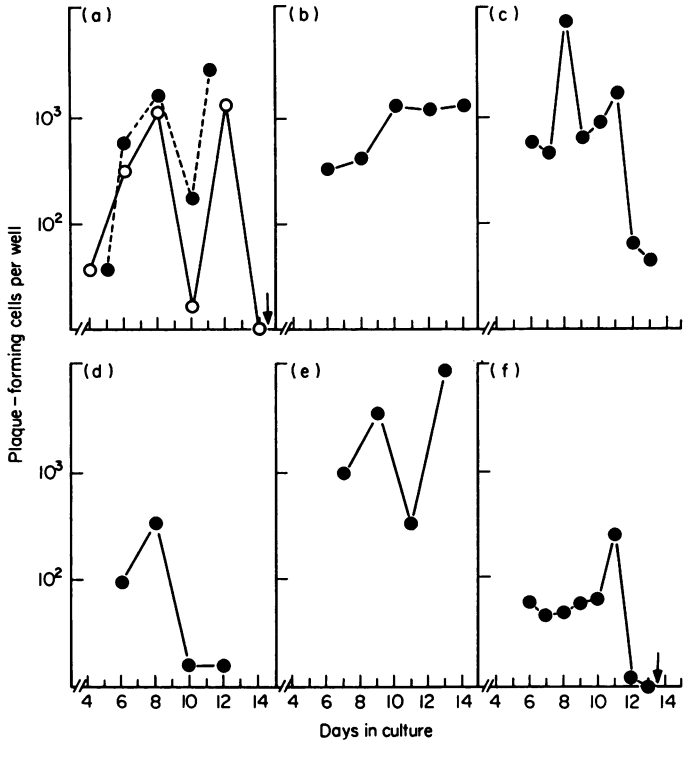


FIG. 1. Kinetics of the antibody response *in vitro* to SRBC of PBL from six different donors. In (a) results were obtained with the same donor in two experiments performed with an interval of 4 months. ↓ = Zero PFC.

instances they could be observed for a period of a week or more. Sometimes a second peak of PFC arose beyond day 10 (Fig. 1). The variability between different donors was in general more pronounced than the variability between different experiments with the same donor (Fig. 1a).

The plaques observed throughout the culture period were all of the direct type.

*Magnitude of the response in normal donors*

Significant differences in the capacity to mount a response were observed among different donors. A graphic representation of the results obtained with thirty-six donors, assayed repeatedly over a period of more than a year, is given in Fig. 2. The results are derived from forty-four experiments for which eight different batches of virus of similar activity were used successively. The values given are the maximum number of PFC per culture well observed before day 9 in each experiment.

In spite of the fact that a detailed kinetics study was not performed in all the experiments, we believe that the values give an indication of the magnitude of the early appearing (first peak) response. The eventual appearance of a second burst of PFC is not, however, taken into account. Donors were ranked from low- to high-responsiveness on the basis of the highest values.

Although the overall range of response covered four orders of magnitude, each donor had a much more limited scatter (rarely exceeding one order of magnitude). It should be noted that almost all the negative data derive from one experiment (AT 78: open circles) in which only one donor responded. The number of plaques observed with this positive donor was in the expected range, although lower than in the other three experiments in which this individual was tested. No obvious technical reasons seem to account for the low results and the batch of virus used in experiment AT: 78 was successfully used on other occasions. For these reasons the data were not excluded from the tabulation.

Table 2 gives some serological data obtained on three 'high' and two 'low' responders. None of the five

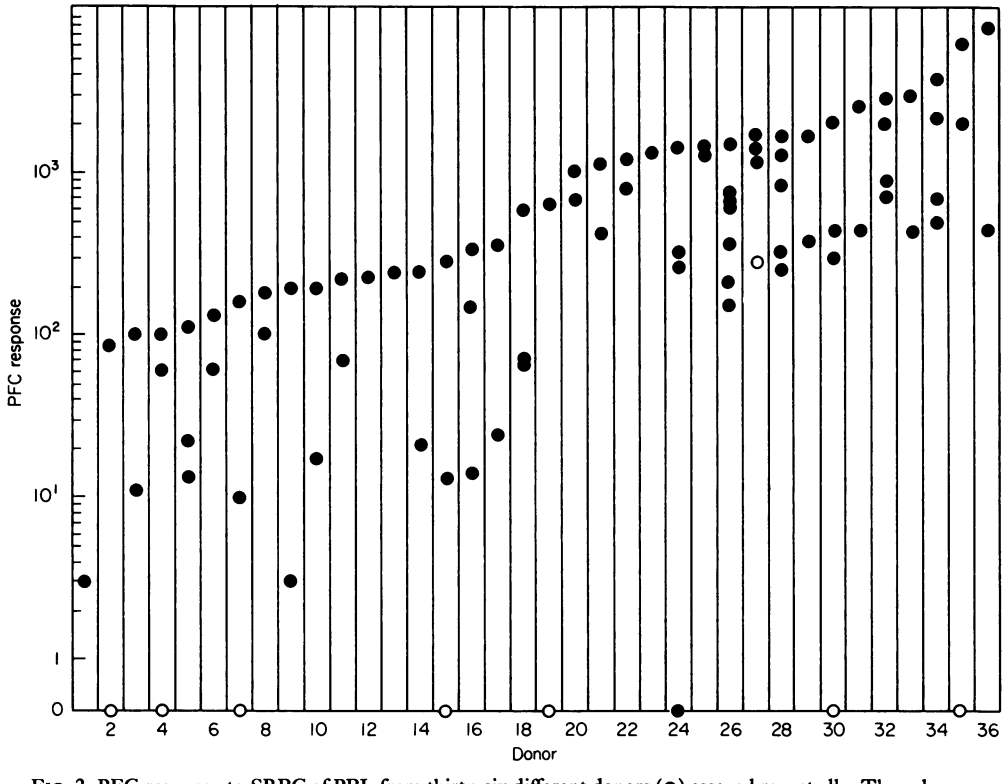


FIG. 2. PFC response to SRBC of PBL from thirty-six different donors (●) assayed repeatedly. The values are the maximum number of PFC per culture well observed before day 9 in each experiment. The donors are ranked according to increasing responsiveness on the basis of the highest values. (○) Results obtained in experiment AT:78 (see Results section).

donors had heterophil antibodies or an appreciable level of IgM antibodies to EBV. This indicates that active EBV infection was not present at the time of the bleeding. Three of the donors had a significant titre of IgG antibodies to EBV, indicating previous exposure to the virus, but these values do not correlate with the capacity of the donors to mount an anti-SRBC response *in vitro*.

No correlation was found between height of the *in vitro* response and age and sex of the donors. An excess of HLA-A9 was observed among the higher responders (unpublished results).

TABLE 2. Lack of correlation between magnitude of the response *in vitro* and presence in the serum of heterophil or anti-EBV antibodies

| Donor | Anti-EBV* IgM | IgG | HA† | PFC per well Day 7 | Day 9 |
|---|---|---|---|---|---|
| HC | < 16 | 64 | Neg. | 1660 | 380 |
| HH | < 16 | 64 | Neg. | 486 | 630 |
| BV | < 16 | < 16 | Neg. | 70 | 20 |
| BE | < 16 | < 16 | Neg. | 1045 | 3800 |
| TS | < 16 | 32 | Neg. | 16 | 22 |

* Titre expressed as reciprocal of serum dilution: indirect immunofluorescence test (Henle & Henle, 1966).

† Heterophil antibodies (Paul–Bunnell).

TABLE 3. Infectious mononucleosis patients: serological data and antibody response *in vitro*

| | | Anti-EBV* IgM | Anti-EBV* IgG | HA† | PFC‡ |
|---|---|---|---|---|---|
| Patient WM | < | | | | |
| First symptoms | 5/6/77 | | | | |
| Expt. 101 | 13/6/77 | 160 | 320 | + | 0 |
| 104 | 21/7/77 | 20 | 160 | + | 3900 |
| 115 | 29/9/77 | Neg. | 160 | Neg. | 243 |
| Patient PM | | | | | |
| First symptoms | 12/6/77 | | | | |
| Expt. 102 | 16/6/77 | 80 | 40 | + | 0 |
| 103 | 14/7/77 | 40 | 40 | ± | 207 |
| 115 | 29/9/77 | Neg. | 40 | Neg. | 330 |
| Patient HE | | | | | |
| First symptoms | 1/8/77 | | | | |
| Expt. 109 | 25/8/77 | 80 | 80 | + | 18 |
| 110 | 1/9/77 | 80 | 40 | + | 0 |
| 113 | 20/9/77 | 40 | 80 | ± | 170 |
| Patient BL | | | | | |
| First symptoms | 9/6/77 | | | | |
| Expt. 102 | 16/6/77 | 160 | 320 | + | 0 |
| 118 | 20/10/77 | 10 | 40 | ± | 1400 |

* Titre expressed as reciprocal of serum dilution: indirect immunofluorescence test (Henle & Henle, 1966).

† Heterophil antibodies (Paul–Bunnell).

‡ PFC to SRBC after 8 days of culture. Note that all the experiments with IM patients were performed with one batch of virus, which was also tested in several experiments with normal donors.

*Response in infectious mononucleosis patients*

Four cases of IM were studied. During the acute stage of the disease they all had the typical blood picture, characterized by an increase in lymphocytes and monocytes and by the presence of at least 10% of atypical lymphocytes. Heterophil antibodies and anti-EBV antibodies were present (Table 3). Patient HE (32-years-old) had a more severe form, with the development of icterus.

When PBL from patients in the acute stage of the disease were cultured with EBV and SRBC, no PFC to the antigen could be observed throughout the culture period (up to day 11). Tests for indirect PFC (IgG) and controls with SRBC or EBV alone were also negative. However, when tested a few weeks

TABLE 4. *In vitro* response of PBL from recovering IM patients: dependence on antigen and virus

| Donor | Experiment* | Ag | EBV | PFC per well Day 6 | PFC per well Day 8 |
|---|---|---|---|---|---|
| WM | 104 | − | + | 223 | 126 |
| | | + | + | 189 | 3900 |
| PM | 103 | − | + | 16 | 37 |
| | | + | + | 64 | 207 |
| | | + | − | 0 | 0 |
| BL | 118 | − | + | 96 | 171 |
| | | + | + | 1032 | 1400 |

* See Table 3

later, all the patients responded (Table 3). The capacity to respond *in vitro* returned to normal in a stage of the disease in which heterophil and anti-EBV antibodies were still detectable in the serum. As seen in the normal donors, the induction of the antibody response required addition to the culture of both the EBV and the antigen (Table 4).

## DISCUSSION

The data reported in this paper (a) confirm and extend the early observation (Luzzati *et al.*, 1977) on the effect of EB virus on the induction of a specific antibody response *in vitro* with human cells, (b) indicate that the magnitude of the response obtained with different donors is a constant individual characteristic, and (c) show a lack of responsiveness in the lymphocytes of acute infectious mononucleosis patients.

Successful immunization of normal donors can occur only after removal of adhering inhibitor cells, as was observed previously both for human (Luzzati *et al.*, 1976) and for rabbit (Luzzati & Lafleur, 1976) blood cells.

The time course of appearance of PFC shows some peculiarities, namely a high variability among donors and among experiments, and the presence in several instances of two peaks (at days 7–9 and 11–13). These features could be the results of the interplay of different phenomena, i.e., (1) an antigen- and virus-induced immune response, (2) a subsequent or concomitant active transformation of responding cells and (3) a late infection or superinfection of human cells by new virus particles produced in culture. Binding of the EBV to its receptor on B lymphocytes may act, in the presence of antigen, as a stimulatory signal for the induction of a specific immune response. The kinetics of this response may be very similar to that observed earlier for human PBL stimulated with SRBC alone or in the presence of a mouse T cell factor (Luzzati *et al.*, 1976), with a maximum around day 8. The late appearing response could be due to EBV-transformed cells and/or cells infected with newly released virus particles.

It is obvious from the data presented here that the magnitude of the response varies from donor to donor. Variations observed in the PFC number obtained from the same donor in different experiments may have a kinetic basis, in that the peak response may not always have been measured. Moreover, exceedingly low numbers of PFC obtained in a few instances, could be due to technical reasons (as is probably the case for experiment AT:78 in Fig. 2), or to a transient hypo-responsiveness of the donor, as demonstrated by the data obtained with infectious mononucleosis patients.

In spite of these variabilities, the overall picture shows a remarkable consistency within each donor. As the PFC response depends on two stimuli, one given by the antigen and one given by the EBV, the individual variability observed could be due to differences in the response to the virus or to the antigen.

No correlation was observed between the magnitude of the response *in vitro* and the serum titre of the IgG anti-EBV antibodies, which are the the expression of previous exposure to the virus. On the other hand, adult T cells were shown to be able to inhibit B cell proliferation by EBV (Thorley-Lawson, Chess & Strominger, 1977). Although individual variations were not reported, it is possible that some of the heterogeneity in the magnitude of the response observed in our experiments was due to different proportions of suppressor cells.

Individual variations in antibody titre following immunization have been reported for several species and antigens. For antigens with multiple determinants, e.g. the SRBC used in the present work and the bacterial antigens, high and low responses are determined by the cumulative effect of several genes (Biozzi *et al.*, 1970; Eichmann, Braun & Krause, 1971). There is evidence in mice that some of the genes controlling the response to SRBC may be H2 linked (Ando & Fachet, 1977). We have HLA typed the human donors used in the present work and we have observed an excess of HLA-A9 among the higher responders. Whether this excess is fortuitous must await further experiments.

When PBL from IM patients, infected *in vivo* with the virus and actively producing antibodies to SRBC, are cultured in the system described here in the presence of EBV and SRBC, an enhanced response, perhaps of the secondary type, might be expected. On the contrary, however, a complete unresponsiveness during the acute stage of the disease was consistently observed.

Humoral immune responses are mostly elevated in IM, as demonstrated by the increased immuno-

globulin level and by the presence of high amounts of antibodies of various specificities (Carter, 1975). This virus-induced hyperstimulation of the immune system may affect the capacity of the B cells to respond *in vitro* to a further stimulation by the virus or by the antigen. It is also possible that at the time of acute illness, precursor B cells specific for SRBC are depleted from the circulating lymphocytes and localized in lymphoid organs. On the other hand, effector T cells specific for EBV-genome-carrying B cells were demonstrated in the PBL of patients with acute IM (Svedmyr & Jondal, 1975). Such cells may be the cause of the unresponsiveness observed when stimulating *in vitro* acute IM lymphocytes with antigen.

Alternatively, T cells from IM patients in the acute stage of the disease may provide insufficient help for the *in vitro* antibody response. In fact, although the absolute number of T cells is increased in this stage of the disease (Papamichail, Sheldon & Holborow, 1974), T cell functions have been shown to be generally impaired (Haider, Coutinho & Edmond, 1973; Mangi *et al.*, 1974).

Finally, we cannot exclude the possibility that T cells of IM patients have different adherence properties as compared to normal PBL. This may result in an unfavourable balance of helper and suppressor cells following the nylon wool treatment, which is routinely performed for all the donors.

During recovery, PBL from patients regain the capacity to respond *in vitro*, thus showing that the unresponsive state is only temporary.

The results presented here indicate that the *in vitro* system used allows the detection of individual differences in the capacity to respond to an antigen and of variations caused by a clinical condition. Therefore, we believe that this method may become a suitable tool for studies of immune responses in man.

We are very grateful to the physicians who provided the blood from infectious mononucleosis patients; the blood donors who very generously contributed the material essential for this work; Dr J. Stocker who performed the HLA typing and mediated between ourselves and the blood donors; Prof. U. Krech (Institut für med. Mikrobiologie des Kantons St Gallen) who performed the serological tests.

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