Epstein–Barr (EB) Virus Genome-containing, EB Nuclear Antigen-negative B-lymphocyte Populations in Blood in Acute Infectious Mononucleosis

By D. H. CRAWFORD, A. B. RICKINSON, S. FINERTY AND M. A. EPSTEIN

Department of Pathology, The Medical School, University of Bristol, Bristol, U.K.

(Accepted 19 September 1977)

SUMMARY

Experiments have been performed to identify the type and size of cell infected by EB virus in the blood of acute infectious mononucleosis (IM) patients, and to investigate the nature of the infection. Virus-infected cells, recognized by their ability to give rise to lymphoblastoid cell lines when co-cultivated with foetal lymphocytes, were shown to be restricted to the B-lymphocyte population. Samples of this population from each of eight IM patients were found to be negative for EB nuclear antigen (EBNA) staining. Thereafter, fractions of IM B-lymphocytes prepared on the basis of cell size were assayed either by co-cultivation, for the incidence of virus-infected cells, or by immunofluorescence staining for the presence of cells expressing EBNA. The great majority of virus-infected cells were found in the fractions of normal sized B-lymphocytes and yet these fractions were unequivocally EBNA-negative. The finding of EB virus-infected, EBNA-negative B-cell populations in IM blood is discussed in terms of the type of infection established by EB virus in the circulation of IM patients.

INTRODUCTION

Paul–Bunnell positive infectious mononucleosis (IM) is a benign, self-limiting lymphoproliferative disease known to be caused by the Epstein–Barr (EB) virus (Henle, Henle & Diehl, 1968). EB virus-infected cells are clearly present in the blood of IM patients since leucocytes cultured from this source readily give rise to virus genome-containing cell lines of B-lymphocyte type (Pope, 1967; Diehl et al. 1968; Jondal & Klein, 1973). Since, in vitro, EB virus can confer on B-lymphocytes the ability to give continuous cell lines (Henle et al. 1967; Pope, Horne & Scott, 1968; Pattengale, Smith & Gerber, 1973) and in vivo is suspected of playing a causative role in two human malignancies, Burkitt’s lymphoma and nasopharyngeal carcinoma (Epstein & Achong, 1973b; Henle & Henle, 1973; Klein, 1975), it has been widely assumed that the virus-infected cells in IM blood are potentially malignant B-lymphocytes transformed by the virus to be capable of direct outgrowth to give cell lines when placed in culture (see for instance Klein, 1973–4).

Objections to this view (Epstein & Achong, 1973a) were supported by a series of experiments showing that IM-derived cell lines do not arise by direct outgrowth but by a quite different two-step process (Rickinson et al. 1974, 1975; Rickinson, Finerty & Epstein, 1977) in which cells latently infected by the virus in vivo are activated to a cycle of virus production
in vitro and release virus particles which then infect and transform B-lymphocytes co-resident in the culture to give the permanent cell lines.

Thus, most of the virus-infected cells in IM blood do not appear to be transformed by the virus since there was no evidence of their independent in vitro outgrowth; however, the results did not exclude the possibility that a small minority of these cells might indeed be transformed and capable of direct growth in vitro, but that their contribution to the overall yield of proliferating cells in the cultures was so small that it had gone unnoticed (Rickinson et al. 1974, 1975).

The present work was therefore undertaken to determine first of all which of the various sub-populations of leucocytes from IM blood was infected by EB virus in vivo, and thereafter, a search was made within this population for the presence of a small number of transformed cells capable of direct in vitro growth.

**METHODS**

**Blood donors**

*IM patients.* Heterophil antibody-positive IM patients from the general Bristol population were bled within 8 weeks of the onset of symptoms. Blood (50 ml) was taken by venepuncture into a heparinized syringe [20 international units (iu) heparin/ml]; a small volume of plasma from this blood was used for EB virus antibody studies. Ethical considerations prevented larger quantities of blood being taken from the patients.

*Foetal-cord blood.* Blood samples (20 to 50 ml) were taken from full term human placentae by venepuncture, and transferred into sterile, heparinized bottles (20 iu heparin/ml).

**Cell counts.** Total and differential leucocyte counts were done by standard methods.

**Medium.** RPMI 1640 medium supplemented with 2 mM-glutamine, 100 iu/ml penicillin and 100 μg/ml streptomycin was used for all washing procedures during the preparation of cell samples; 15% foetal calf serum (FCS) was added to this medium for culturing purposes. Leibovitz L15 medium containing phenol red indicator (3 ml/l) and supplemented with 2 mM-glutamine, 200 iu/ml penicillin, 200 μg/ml streptomycin and 2% FCS was used throughout the velocity sedimentation procedure.

**Purification of cell types**

1. **Platelets, granulocytes, mononuclear cells.** Blood samples diluted 1:2 with phosphate-buffered saline were centrifuged on a Ficoll–Triosil density gradient to obtain purified platelets (Pope, Scott & Moss, 1973) and purified granulocytes and mononuclear cells (Böyum, 1968); the mononuclear cell population consisted of monocytes, T-lymphocytes and B-lymphocytes.

2. **T-lymphocytes, B-lymphocytes with monocytes, purified B-lymphocytes.** In some experiments T-lymphocytes were purified from the mononuclear cell population by allowing them to form E-rosettes with sheep erythrocytes (Wybran, Chantler & Fudenberg, 1973) and then separating the rosetted and non-rosetted fractions by a second Ficoll–Triosil centrifugation (Greaves & Brown, 1974). Preparations of T-lymphocytes were obtained from the rosetted fraction by lysis of sheep erythrocytes in ammonium chloride solution (Jondal, 1974a); the preparations contained less than 10% surface immunoglobulin-positive cells as determined by immunofluorescence testing (Fröland, Natvig & Berdal, 1971). The non-rosetted fraction provided preparations of B-lymphocytes with monocytes in which up to 95% of the cells were surface immunoglobulin-positive; in further experiments, purified
EB virus-infected EBNA-negative cells in IM

B-lymphocytes were prepared from this material by removing phagocytic cells (monocytes) with a magnet following incubation with carbonyl iron particles (Jondal, 1974b)

(3) Adherent and non-adherent mononuclear cells. In other experiments the method of Pope et al. (1973) provided an adherent fraction, consisting of 85 to 95% monocytes and 5 to 15% lymphoid cells, and a non-adherent fraction containing 95 to 100% lymphoid cells.

Each purified cell fraction was washed twice and the cells were counted.

Cell separation by size using velocity sedimentation. The method adopted was essentially that described by Miller & Phillips (1969) as adapted for the separation of IM mononuclear cells by Denman & Pelton (1973, 1974). In order to ensure adequate separation, $5 \times 10^7$ to $2 \times 10^8$ cells were loaded into the sedimentation chamber above a buffered step gradient of 0.33% to 2% Ficoll in L15 medium; if the total number of cells to be separated was less than $5 \times 10^7$, washed sheep erythrocytes were used as a supplement in the initial loading of the chamber. Fractions (25 ml) were collected from the base of the chamber after 4 h sedimentation and numbered 1 to 22; since cells fall through the gradient at a rate proportional to the square of their radius, the largest cells appeared in the earliest fractions. The cells in each fraction were centrifuged to a pellet, resuspended in 1 ml medium and counted.

Assessment of cell populations. To assess the homogeneity of the composition of the various cell populations, differential leucocyte counts were done on slide preparations in conjunction with size measurements made on photomicrographs.

Culturing techniques. Cells were cultured in 2 ml of medium in 1.5 cm diameter plastic vials (Sterilin, Richmond, Surrey) in a humidified atmosphere of 5% CO$_2$ in air. The cultures were re-fed every 10 days by replacing half the medium without disturbing the cell layer, and were observed regularly over a period of at least 10 weeks for signs of transformation. Transformation was recognized by the presence of foci of actively growing cells which continued to proliferate after sub-culturing.

Examination of chromosomes. Chromosome spreads were prepared from cultures containing transformed cells by methods already described (Jarvis et al. 1974) and were stained with Giemsa.

The sex distribution within each cell population was determined by establishing the sex of between 10 and 20 spreads from each culture.

Virus capsid antigen (VCA) staining. The EB virus-antibody status of the IM patients was determined by using their plasma samples in an indirect fluorescence test to detect antibodies to VCA (Henle & Henle, 1966).

EBNA staining. EBNA staining was performed as described by Reedman & Klein (1973) with the added complement reinforcement step suggested by Klein et al. (1976). Duplicate slides of each cell preparation were stained using known anti-EBNA positive sera of high titre or known negative sera. On each occasion EBNA-positive Raji cells (Pulvertaft, 1964) and EBNA-negative Ramos cells (Klein et al. 1975) were included as controls. In addition, the sensitivity of the EBNA staining technique was assessed by including, as further controls, preparations made from mixtures of Raji and Ramos cells at ratios down to 1:100, preparations made from foetal lymphocytes 2 days after their infection with EB virus in vitro, and preparations made from fractions obtained when mixtures of Raji cells and foetal lymphocytes were separated on the basis of cell size using the velocity sedimentation technique.

Experimental procedure. A first group of experiments was designed to identify EB virus-
infected cells in leucocyte sub-populations prepared from IM blood. Virus-infected cells
were detected by their ability to give rise to cell lines when co-cultivated with foetal cells.
Mononuclear cells (4 \times 10^6), granulocytes, B-lymphocytes with monocytes, or T-lymphocytes
from IM blood were each co-cultivated with an equal number of foetal mononuclear cells
of the opposite sex; the platelets from each IM patient were centrifuged into two equal
pellets, each of which was likewise co-cultivated with 4 \times 10^6 foetal mononuclear cells. In
separate experiments within this group, adherent and non-adherent cell fractions were
prepared from 10^7 IM mononuclear cells, and each fraction was co-cultivated with 4 \times 10^6
foetal mononuclear cells in the same way. Cultures showing signs of transformation within
the 10-week observation period were sub-cultured, and the transformed cells were submitted
for chromosome analysis to determine their donor origin. Control cultures of foetal mono-
nuclear cells alone were always included.

Since subsequent work involved experiments in which leucocytes were separated into
fractions on the basis of size, a preliminary assessment of the quality of the size separation
was next made. Cells in fractions separated by size from IM mononuclear cell populations
or from B-lymphocytes with monocytes were counted and measured.

The experiments of the second group were designed to detect the presence of virus-
infected cells in fractions of IM B-lymphocytes with monocytes separated by size. Each of
22 fractions was co-cultivated with 4 \times 10^6 foetal mononuclear cells of the opposite sex,
although in some cases consecutive fractions were pooled or subdivided, where necessary,
to give around 10^6 IM-derived cells per co-culture. As before, cultures showing transforma-
tion during 10 weeks of observation were sub-cultured and the transformed cells sexed to
determine their donor origin. Foetal control cultures were likewise included.

In a third group of experiments the presence of EBNA-positive cells was sought in the
circulating B-lymphocyte populations of a further set of IM donors. In each case, prepara-
tions for EBNA staining were made both from a sample of the purified B-lymphocyte
population before fractionation and from the 22 fractions obtained when these cells were
separated on the basis of size; consecutive fractions were pooled to give 1 to 2 \times 10^6 cells
per preparation, the pooled preparations being fractions 1 to 8, 9 and 10, 11 and 12, 13 and
14, 15 and 16 and 17 to 22. Each preparation was examined after EBNA staining by three
independent observers. In most experiments, the staining procedure was repeated on a second
occasion using duplicate slides and a different pair of high titre EBNA-positive and control
EBNA-negative sera.

RESULTS

Table 1 shows the time after onset of symptoms at which blood was taken from the
IM patients, together with the results of the blood counts and EB VCA titrations in each
case. It can be seen that the patients used for each group of experiments were strictly com-
parable by these criteria.

The results of the various experiments of the first group using blood samples from twelve
IM patients are presented in Table 2, which shows the overall incidence of transformation and
the mean time to transformation for each type of co-culture set up. Transformed foci ap-
peared after a mean time of 44 days in approximately half of those co-cultures containing
all the IM mononuclear cells and, after a mean time of 34 days, in almost every one of those
containing only B-lymphocytes with monocytes. In contrast, none of the co-cultures
containing T-lymphocytes, granulocytes or platelets showed any signs of transformation.
The results of three further experiments, in which the IM mononuclear cells were separated
EB virus-infected EBNA-negative cells in IM

Table 1. Haematological data on IM donors* of blood used in the experiments

<table>
<thead>
<tr>
<th>IM patient number</th>
<th>Age (years)</th>
<th>Duration of symptoms at bleeding (weeks)</th>
<th>Total $\times 10^3$/ml</th>
<th>Differential leucocyte count (%)</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total granulocyte</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Monoocyte</td>
<td>Atypical</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>1</td>
<td>15</td>
<td>70</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>4</td>
<td>7.7</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>1</td>
<td>9.1</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
<td>3</td>
<td>5.8</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>4</td>
<td>5.7</td>
<td>42</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>2</td>
<td>6.5</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>3</td>
<td>4.7</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>8</td>
<td>6.5</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>2</td>
<td>9.4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>23</td>
<td>2</td>
<td>9.7</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>2</td>
<td>5.4</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>13</td>
<td>19</td>
<td>3</td>
<td>ND</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>18</td>
<td>3</td>
<td>ND</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>18</td>
<td>4</td>
<td>ND</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>19</td>
<td>1.5</td>
<td>ND</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>17</td>
<td>16</td>
<td>2</td>
<td>9.2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>18</td>
<td>24</td>
<td>2</td>
<td>6.5</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>3</td>
<td>5.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>23</td>
<td>2</td>
<td>8.4</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>19</td>
<td>2</td>
<td>9.6</td>
<td>61</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>23</td>
<td>8</td>
<td>6.5</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>23</td>
<td>38</td>
<td>3</td>
<td>9.4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>3</td>
<td>4.5</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>25</td>
<td>19</td>
<td>0.5</td>
<td>16</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>30</td>
<td>3</td>
<td>7.6</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>27</td>
<td>45</td>
<td>3</td>
<td>5.1</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>28</td>
<td>16</td>
<td>3</td>
<td>8.1</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>29</td>
<td>20</td>
<td>2</td>
<td>8.0</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>2</td>
<td>13</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>31</td>
<td>16</td>
<td>1</td>
<td>8.0</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>32</td>
<td>19</td>
<td>2</td>
<td>7.0</td>
<td>18</td>
<td>12</td>
</tr>
</tbody>
</table>

* All patients were VCA antibody-positive to a dilution of at least 1/16.
† ND = Not done.

into adherent and non-adherent fractions before co-cultivation with foetal cells, are presented in Table 3. Transformation was not seen in co-cultures containing the adherent fraction whereas transformed foci did appear in some co-cultures containing the non-adherent cells, although at a lower incidence than was seen in parallel co-cultures containing the corresponding total unfractionated IM mononuclear cell population.

In the experiments to assess the quality of fractions separated by size, it was found that fraction 1 contained cells with an average diameter of 20 µm, 80% of which were atypical lymphocytes together with some eosinophils; this, and neighbouring large cell fractions included all the monocytes. In fraction 10, 90% of the cells were medium sized lymphocytes with an average diameter of 10 µm, and in fraction 22 almost all the cells were very small lymphocytes with an average diameter of 7.5 µm. Figure 1 presents typical values for cell yields in each of the 22 size fractions obtained when either total mononuclear cells or B-lymphocytes with monocytes were separated in this way. The total mononuclear cell popula-
Table 2. Incidence of transformation in co-cultures between foetal mononuclear cells and purified sub-populations of leucocytes from acute IM blood*

<table>
<thead>
<tr>
<th>Type of IM cells in culture</th>
<th>Incidence of transformation</th>
<th>Mean time to transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononuclear cells</td>
<td>11/24</td>
<td>44 days</td>
</tr>
<tr>
<td>B-lymphocytes with monocytes</td>
<td>18/19</td>
<td>34 days</td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td>0/60</td>
<td>-</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>0/6</td>
<td>-</td>
</tr>
<tr>
<td>Platelets</td>
<td>0/10</td>
<td>-</td>
</tr>
</tbody>
</table>

* Results are from 12 IM patients numbered 1 to 12.

Table 3. Incidence of transformation in co-cultures between foetal mononuclear cells and either unfractionated IM mononuclear cells or their adherent and non-adherent sub-populations*

<table>
<thead>
<tr>
<th>Type of IM cells in culture</th>
<th>Incidence of transformation</th>
<th>Mean time to transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated mononuclear cells</td>
<td>6/9</td>
<td>45 days</td>
</tr>
<tr>
<td>Adherent mononuclear cells</td>
<td>0/9</td>
<td>-</td>
</tr>
<tr>
<td>Non-adherent mononuclear cells</td>
<td>3/9</td>
<td>50 days</td>
</tr>
</tbody>
</table>

* Results are from three IM patients numbered 13 to 15.

...
total population of foetal lymphocytes 2 days after infection with EB virus in vitro. As a final control, when mixtures of Raji cells and freshly prepared foetal lymphocytes were separated on the basis of cell size by velocity sedimentation, Raji cells were found concentrated within the first few fractions to be collected where they were clearly identifiable by the EBNA staining technique.

**DISCUSSION**

The results of the first group of experiments clearly demonstrate that EB virus-infected cells in vivo in acute IM blood are restricted to the B-lymphocyte population (Tables 2 and 3), exactly as would be expected from in vitro studies which have shown that only B-lymphocytes have surface receptors for EB virus (Jondal & Klein, 1973; Greaves, Brown & Rickinson, 1974; Jondal et al. 1976).

The remaining experiments were designed to clarify the nature of the infection established by EB virus in vivo in the B-lymphocyte of IM patients. When EB virus infects B-lymphocytes in vitro, it induces cellular DNA synthesis (Gerber & Hoyer, 1971) and the appearance of large EBNA-positive blast cells transformed by the virus to be capable of unlimited proliferation (Leibold et al. 1975; Moss & Pope, 1975). By analogy, therefore, it might be expected that the virus-infected cells in IM blood are present amongst the large atypical lymphocytes which are characteristic of this disease (Downey & McKinlay, 1923; Finch, 1969) and furthermore, that they are similarly transformed by the virus, potentially malignant but held in check by immunological surveillance (Klein, 1973-4). Although many of the atypical lymphocytes in IM have been identified as T-cells (Sheldon et al. 1973) a small minority is of B-cell origin (Enberg, Eberle & Williams, 1974; Papamichael, Sheldon &
Table 4. *Occurrence of transformation in co-cultures between foetal mononuclear cells and fractions prepared on the basis of cell size from IM B-lymphocytes with monocytes*

<table>
<thead>
<tr>
<th>IM patient number</th>
<th>I</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>I</td>
<td>+</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* + = Transformation observed; - = no transformation observed; I = culture infected and discarded.

Table 5. *Origin of transformed cells, as shown by chromosome analysis, in co-cultures between foetal mononuclear cells and fractions prepared on the basis of cell size from IM B-lymphocytes with monocytes*  

<table>
<thead>
<tr>
<th>Fractions prepared from IM B-lymphocytes with monocytes</th>
<th>Origin of transformed cells (%) from 20 spreads per co-culture</th>
<th>IM donor: Foetal donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7</td>
<td></td>
<td>0:100</td>
</tr>
<tr>
<td>8-10</td>
<td></td>
<td>25:75</td>
</tr>
<tr>
<td>11-12</td>
<td></td>
<td>10:90</td>
</tr>
<tr>
<td>13-14</td>
<td></td>
<td>50:50</td>
</tr>
<tr>
<td>15-16</td>
<td></td>
<td>25:60</td>
</tr>
<tr>
<td>17-22</td>
<td></td>
<td>0:100</td>
</tr>
</tbody>
</table>

* Results are from seven IM donors numbered 18 to 24.
EB virus-infected EBNA-negative cells in IM

Holborow, 1974) and it is within this B-cell population that the presence of a few EBNA-positive cells has recently been reported (Klein et al. 1976).

In the second group of experiments, the incidence of transformation in the co-cultures of foetal cells and B-lymphocytes with monocytes fractionated on the basis of cell size, provided a measure of the relative proportion of EB virus-infected cells in the different fractions. This must relate directly to the distribution of infected cells across the spectrum of cell size as it exists in IM blood, since the co-cultures were set up within 6 h of bleeding the patient, long before in vitro activation of infection and intercellular transfer of newly synthesized virus particles could have occurred (Rickinson, Epstein & Crawford, 1975). Since transformation was seen most regularly in the co-cultures containing fractions 11 to 14 (Table 4), the majority of virus-infected cells in IM blood must reside in the population of normal sized B-lymphocytes. In some experiments foci of transformed cells appeared in co-cultures containing a broader range of fractions running from 7 to 17, and in one single case (IM patient 18, Table 4), transformation was seen in virtually all the co-cultures. This indicates that in certain patients at least, virus-infected cells can be detected amongst lymphoid cells of all sizes; it may be that such patients are at a clinically more acute stage of the disease with a relatively larger proportion of the circulating cells infected by the virus.

Of the EB virus-determined antigens detectable by fluorescent antibody techniques, EBNA is the only one consistently expressed in virus-transformed cells capable of direct outgrowth to give cell lines in vitro (Reedman & Klein, 1973). Accordingly, in the final group of experiments EBNA-positive cells were sought in the total population of purified IM B-lymphocytes, and in fractions of this population prepared on the basis of cell size as well, in order to relate the incidence of EBNA-positive cells to the incidence of virus-infected cells. Because of the constraints on the amount of blood which could be taken from each patient and the limited numbers of B-lymphocytes obtainable from any one IM blood sample, the detection of virus-carrying cells and the EBNA testing had to be done on blood samples from separate groups of patients. However, the patients in the two groups were strictly comparable in terms of duration of symptoms and blood picture (Table 1).

The results of the EBNA tests call for comment. In our hands the EBNA staining technique was sufficiently sensitive to detect isolated EBNA-positive cells where these were present in populations at concentrations of between 0.1 and 1%. The failure to find EBNA-positive cells amongst the B-lymphocytes from eight IM donors despite careful analysis of the preparations by three separate observers is of considerable significance because this result is at variance with that of Klein et al. (1976). They reported that large EBNA-positive blast cells were present in the circulation of four out of seven acute IM patients examined, where they constituted 0.5 to 2% of the B-cell population. If this number of cells had been present in the blood of the eight patients used in the present work they would have appeared in the fractions of larger cells (fractions 1 to 8) where they would have been concentrated to an easily detectable level. It might be that the eight patients free of EBNA-positive cells studied here correspond to the three similar patients reported by Klein et al. (1976) and that the positive EBNA expression reported in four patients of the latter series merely denoted cells in the early stages of a productive infectious cycle.

In any event, it is clear from the present experiments that in IM the EB virus-infected cells in the circulation are B-lymphocytes (Tables 2 and 3), mainly of normal size (Table 4), which do not express EBNA in vivo, yet which give rise to cell lines when co-cultivated with foetal cells in vitro (Table 4). Chromosome analysis of the cell lines which appeared in the co-cultures showed that the two-step process of virus release followed by infection and transformation of co-resident cells took place on a large scale, since cell lines of foetal
origin were either exclusively or predominantly present (Table 5). There can be no doubt that this virus was released following activation in vitro of a productive infection in cells which had been carrying the virus genome in a non-infectious form in vivo; infectious virus cannot be detected either adsorbed to the surface or sequestered within leucocytes taken direct from the patient and is only manifest after the cells have been placed in culture for a few days (Rickinson, Epstein & Crawford, 1975).

The finding of the present work that the EB virus genome-containing cells in IM blood are EBNA-negative, and therefore seemingly not transformed by the virus, raises yet again the important question as to the real nature of their infection. On the one hand these cells may be able to persist in vivo harbouring the virus genome as a latent infection similar to that shown by other herpesviruses (Bastian et al. 1972, 1974; Baringer, 1974) including now a lymphotrophic member of the group – mouse cytomegalovirus (Olding, Kingsbury & Oldstone, 1976); on the other hand these cells may all be in very early stages of the infectious cycle and about to be destroyed once they are recognized by the immune response. T-lymphocytes in IM blood are known to be mounting a specific cell-mediated response to cells bearing the EB virus-associated lymphocyte-detected membrane antigen, LYDMA (Royston et al. 1975; Svedmyr & Jondal, 1975; Rickinson, Crawford & Epstein, 1977), and it seems likely that LYDMA is expressed on at least some, if not all, of the virus-infected cells which can be found in the blood at this time. If this indeed proves to be the case, the present results showing these cells to be EBNA-negative would suggest that the expression of LYDMA precedes that of EBNA in the early stages of the infectious cycle.

This study was assisted by the Cancer Research Campaign, London, England, out of funds donated by the Bradbury Investment Co., Hong Kong. One of us (DHC) was in receipt of a Junior Research Fellowship from the Medical Research Council, London, England. The authors are most grateful to Helen McCallum and Sally Bigwood for excellent technical assistance and to Dr I. D. Fraser, South West Regional Transfusion Centre, Southmead, Bristol, Dr G. L. Scott, Department of Haematology, Bristol Royal Infirmary, Dr G. H. Sylvester and his staff at the University of Bristol Students’ Health Service, and to practitioners in the Bristol area for access to patients.

REFERENCES


EB virus-infected EBNA-negative cells in IM

459


(Received 22 June 1977)