Immunofluorescence in Cells Derived from Burkitt’s Lymphoma

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ABSTRACT

HENLE, GERTRUDE (Children’s Hospital of Philadelphia, Philadelphia, Pa.), AND WERNER HENLE. Immunofluorescence in cells derived from Burkitt’s lymphoma. J. Bacteriol. 91:1248-1256. 1966.—Indirect immunofluorescence tests led to the brilliant staining of a small proportion of the cells in five different cultures derived from Burkitt’s (African) lymphomas. The reaction was not restricted to the 17 sera from cases of this disease but extended to many sera from American individuals, whether healthy donors or patients suffering from a variety of illnesses. The incidence of positive sera increased with age from about 30% in childhood to >90% in adults. Fluorescein-isothiocyanate-conjugated human γ-globulins were suitable for direct staining of the same proportion of cells. The stained cells appeared to be in varying stages of degeneration, but cultural conditions leading to an increase in the cellular death rates failed to result in a rise in fluorescent cells. Several observations suggest that the stainable cells might be those which are seen to harbor virus particles under the electron microscope. Two cell lines derived from leukemic patients in this country also contained a small fraction of stainable cells but two others, and numerous primary human leukocyte cultures, gave consistently negative results. Attempts to relate the staining to known viral antigens have failed to implicate herpes simplex, varicella, cytomegal, and reo viruses types 1, 2, and 3. The nature of the virus carried by the lymphoma cells as well as of the staining reactions remains to be determined.

A number of cell lines have been isolated from Burkitt’s (African) lymphomas which are lymphoblastic in character and grow freely in culture media without attachment to the surface of the glass vessel (6–9, 18, 22; Epstein, Barr, and Achong, Wistar Inst. Symp. Monograph No. 4, in press; Rabson et al., Intern. J. Cancer, in press). Five of these six lines have been examined electron microscopically, and the presence of virus particles in a small proportion of the cells has been reported (5, 8, 22; Epstein et al., in press; Rabson et al., in press). These particles resemble herpes simplex virus in shape but seem to be somewhat smaller in size. They were seen in the nuclei and also in the cytoplasm of cells. Infected cells showed as a rule, some degree of degeneration (10). There is an indication that the virus particles may disappear, or at least decrease in number, on prolonged cultivation of the cells (Epstein, personal communication; Rabson et al., in press). Efforts to transmit the virus to other types of cultures, experimental animals, or chick embryos by routine procedures thus far have failed (10). Several of the lines revealed suggestive evidence of a viral infection by means other than electron microscopy in that they were noted to behave like certain viral carrier cultures (12; Henle and Henle, Wistar Inst. Symp. Monograph No. 4, in press); i.e., they showed a considerable degree of resistance to the usually highly cytopathic vesicular stomatitis virus (VSV); they were capable of transmitting resistance to certain human and simian cells on mixed cultivation; and they released into the culture media a factor possessing all the attributes of an interferon. With the availability of sera from patients with Burkitt’s lymphoma, it became possible to study the various cell lines by the indirect immunofluorescence and complement-fixation techniques. Positive results were obtained in both tests, and they were not restricted to sera from African patients with this disease but extended to many
sera from individuals residing in the United States. Neither were positive reactions obtained solely with the cell lines derived from cases of Burkitt's lymphoma, but also with certain lines isolated from leukemic patients in the United States. The results of immunofluorescence will be presented in this communication; those obtained in complement-fixation tests are reported separately (1).

**MATERIALS AND METHODS**

**Cells.** The various Burkitt lymphoma as well as other cell lines used are listed in Table 1 together with the media employed in this laboratory for their cultivation and optimal growth. (The lymphoma lines had been in culture for 9 to 14 months at the start of these experiments. Since they are maintained mainly by dilution in fresh medium, at most preceded by sedimentation of the cells, it seemed inappropriate to signify each feeding by serial passage numbers.) They were kindly furnished by M. A. Epstein, Bland Sutton Institute, Middlesex Hospital, London, England (EB1, EB2, and EB3); Sarah Stewart (SL4) and A. S. Rabson (AL4), National Cancer Institute, Bethesda, Md.; J. T. Grace, Roswell Park Memorial Institute, Buffalo, N.Y. (64-10); and B. Clarkson, Sloan Kettering Institute, New York, N.Y. (SKL-1 and SKL-2). The LK-1D line was isolated in this laboratory.

Primary cultures of peripheral leukocytes from healthy donors were prepared according to the method employed for chromosome studies (16). The method, involving stimulation by phytohemagglutinin, has been slightly modified (Duc-Nguyen and Henle, to be published). Human diploid cells (WI-38) were obtained from the Wistar Institute, Philadelphia, Pa., and human embryonic kidney (HEK) cultures from Flow Laboratories, Rockville, Md., or Microbiological Associates, Inc., Bethesda, Md. HeLa and Earle's strain L cell cultures were prepared from stock lines maintained in this laboratory. Primary rabbit kidney cell cultures were kindly supplied by T. Tokumaru here at the hospital.

**Sera.** For sera from patients with Burkitt's lymphoma, we are indebted to W. Ray Bryan, National Cancer Institute, D. H. Wright, Makerere University College Medical School, Kampala, Uganda, and M. A. Epstein. Sera from children with leukemia, solid tumors, and various metabolic and infectious diseases were furnished by staff members of The Children's Hospital of Philadelphia. Sera from infants with cytomegalic inclusion disease and from mothers with or without complement-fixing antibodies to this virus were kindly supplied by R. M. McAllister, The Children's Hospital of Los Angeles, Los Angeles, Calif., and J. B. Hanshaw, School of Medicine, University of Rochester, Rochester, N.Y. Sera from adult patients with leukemias and cancers were provided by the Virus Research Resources Branch of the National Institutes of Health, Bethesda, Md. Other adult sera were obtained from blood donors and medical students.

**Fluorescein isothiocyanate (FITC)-conjugated γ-globulins.** For the indirect staining technique (20), rabbit anti-human and anti-guinea pig globulin conjugates were prepared by conventional means as described elsewhere (13) or were purchased from Baltimore Biological Laboratories, as were labeled goat anti-rabbit and anti-rhesus monkey globulins. For direct staining (3), preparations of commercial human γ-globulin or of human mumps hyperimmune γ-globulin (Philadelphia Serum Exchange) were coupled with fluorescein isothiocyanate (19). The latter γ-globulin was obtained from donors who had been immunized against mumps by two doses of a formalin-inactivated vaccine. The donors had been subjected thereafter to plasmapheresis every 2 weeks for several months, and the plasma so obtained revealed a continued high titer of antibodies to mumps (21). The FITC-conjugated γ-globulins prepared from the pooled plasmas had been stored at 4°C since 1959. Rabbit anti-herpes simplex virus conjugates were provided by K. Munk, Virologisches Institut der Universität Heidelberg, and monkey anti-murine leukemia (Rauscher) virus conjugate was kindly supplied by M. A. Fink, National Cancer Institute, Bethesda, Md.

**Preparation of cell smears.** Suspended cell cultures were subjected to centrifugation at 600 × g for 10 to

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**Table 1. Cell lines and culture media**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Authors</th>
<th>Medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB1</td>
<td>Burkitt's lymphoma</td>
<td>Epstein and Barr (6)</td>
<td>BME (45%), MEM (45%), FCS (10%)</td>
</tr>
<tr>
<td>EB2</td>
<td>Burkitt's lymphoma</td>
<td>Epstein et al. (5)</td>
<td>BME (90%), FCS (10%)</td>
</tr>
<tr>
<td>EB3</td>
<td>Burkitt's lymphoma</td>
<td>Epstein et al. (in press)</td>
<td>No. 1629 (90%), FCS (10%)</td>
</tr>
<tr>
<td>SL4</td>
<td>Burkitt's lymphoma</td>
<td>Stewart et al. (22)</td>
<td>BME (90%), FCS (10%)</td>
</tr>
<tr>
<td>AL4</td>
<td>Burkitt's lymphoma</td>
<td>Rabson et al. (in press)</td>
<td>No. 199 (90%), FCS (20%)</td>
</tr>
<tr>
<td>64-10</td>
<td>Myeloblastic leukemia</td>
<td>Ikawata and Grace (14)</td>
<td>No. 1629 (90%), FCS (10%)</td>
</tr>
<tr>
<td>SKL-1</td>
<td>Acute monocytic leukemia</td>
<td>Clarkson (to be published)</td>
<td>No. 1629 (90%), FCS (10%)</td>
</tr>
<tr>
<td>SKL-2</td>
<td>Acute lymphoblastic leukemia</td>
<td>Clarkson (to be published)</td>
<td>No. 1629 (90%), FCS (10%)</td>
</tr>
<tr>
<td>LK-1D</td>
<td>Acute lymphocytic leukemia</td>
<td>Armstrong (to be published)</td>
<td>No. 1629 (90%), FCS (10%)</td>
</tr>
</tbody>
</table>

* BME, Basal Medium Eagle; MEM, minimal essential medium (Eagle); FCS, fetal calf serum.
15 min. The supernatant fluids were drained off, and the cells were resuspended in the remaining drops with the aid of a finely drawn-out Pasteur pipette. A small drop of this suspension was placed on a cover slip (6 by 30 mm), evenly distributed over the surface, and allowed to dry thoroughly at 37 C. The cover slips were fixed at room temperature with acetone for 10 min, drained, and dried. The fixed preparations were kept at —20 C until staining was performed.

Other cells (WI-38, HEK, and HeLa) were grown on cover slips in test tubes and infected with herpes simplex, varicella, or cytomegal (Davis strain) viruses. When lesions became evident, the cover slips were removed, washed in phosphate-buffered saline solution (PBS), dried, and fixed as described.

Staining of cover slips. For the direct technique, the cover slips were overlayed with FITC conjugates and incubated in a moist chamber for 1 hr at 37 C. Subsequently, they were washed twice for 10 min in petri dishes containing PBS, agitated by a magnetic stirrer, and then dipped several times into two consecutive beakers containing distilled water. After drying, the cover slips were fixed onto slides by means of a semipermanent mounting medium (20) and examined under ultraviolet illumination (Osmar HBO 200-w lamp) with a Zeiss microscope.

For indirect staining, the cover-slip preparations were first exposed to serum at a dilution of 1:8 or greater for 1 hr at 37 C. After thorough washing, they were overlaid with FITC-conjugated antibodies to globulins of the appropriate species and processed further as described.

Vital staining, by both the direct and indirect methods, differed from the above techniques only insofar as sedimented unfixed cells were taken up in small volumes of the reagents, incubated for 1-hr periods at 37 C and washed by repeated sedimentation and resuspension in PBS. The final suspensions were spread on cover slips, which, after drying, were mounted on slides with or without prior fixation in acetone.

Other techniques are described below.

RESULTS

Indirect immuno-fluorescence. In the first test, plasma obtained from a case of Burkitt's lymphomas treated at the Clinical Center of the National Institutes of Health, and a serum from a leukemic American child as control were used in a 1:8 dilution to stain EB1 and EB2 cells by the indirect method. With the patient's plasma, apple-green fluorescence was noted in about 1% of the cells. As shown in Fig. 1A and B, the staining was somewhat granular, often revealing larger aggregates within the confines of the cells. Many of the cells showed irregular outlines and appeared to be in varying states of disintegration. The control serum failed to mediate any fluorescence; neither did the rabbit anti-human globulin conjugate per se stain any cells except for occasional faint stippling of the type seen in Fig. 1A.

The indirect immunofluorescence technique was subsequently applied to sera from additional patients with Burkitt's tumor as well as to numerous sera from American individuals of varying ages, and also to other Burkitt lymphoma cell lines. The data concerning the reactivity of the various sera in a 1:8 dilution are summarized in Table 2. All of the 17 sera from Burkitt lymphoma patients gave positive results, but indirect immunofluorescence was elicited also with many sera from American individuals. About 30% of the sera from children reacted regardless of whether they were taken from patients with leukemia, metabolic diseases, or infectious diseases. The incidence of positive staining reactions was distinctly higher (up to >90%) with sera of adult persons which were obtained either from patients with malignancies or from healthy donors.

A number of sera were titrated by the indirect immunofluorescence technique as well as in complement-fixation tests (1). The results (Table 3) revealed that sera which were strongly positive in a 1:8 dilution could be diluted as much as 64-fold and still yield definite, though weak, fluorescence. The intensity of staining at low serum dilutions (despite the subjective nature of the grading) seemed to relate well to the titers measured by the staining technique but not to those obtained in complement-fixation tests. Yet, some degree of correlation between the two assay methods seems to be apparent from the summary presented in Table 4, based merely upon positive or negative results at a serum dilution of 1:8. Of the 96 sera studied by both methods, the results agreed in 72% of the cases; i.e., either both tests were positive or both were negative. However, 24% of the sera yielded positive fluorescence in the absence of detectable complement-fixing activity and 4% failed to stain but reacted in the complement-fixation test.

In control experiments, no staining was obtained when the EB cells were exposed first to sera from several adult rabbits, guinea pigs, or rhesus monkeys and then to FITC-conjugated antibodies to homologous or human globulins.

Direct immunofluorescence tests. The frequency of positive reactions with sera of adult persons in the indirect test rendered it likely that FITC-conjugated pooled human γ-globulin would be suitable for the direct method of staining. Three such preparations were available, and all three fulfilled the expectation. Two derived from commercially produced normal γ-globulin gave distinct staining of moderate intensity. The third was prepared from pooled plasmas obtained by biweekly plasmapheresis from a few donors who had been vaccinated against mumps. Since fre-
Fig. 1. Immunofluorescence elicited in cells derived from Burkitt's lymphoma. (A) Indirect stain of EB2 cells by serum from a patient with Burkitt's lymphoma and FITC-conjugated rabbit antibodies to human globulin. (B) The same except with serum from a healthy donor for the primary reaction. (C) Direct staining of EB1 cells by FITC-conjugated human mumps hyperimmune γ-globulin. (D) Direct stain of EB2 cells by FITC-conjugated normal human γ-globulin. × 1,250.

Table 2. Incidence of positive reactions in indirect immunofluorescence tests with EB1 or EB3 cells

<table>
<thead>
<tr>
<th>Age group</th>
<th>Diagnosis</th>
<th>No. of individuals</th>
<th>Fluorescence</th>
<th>Per cent +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>Burkitt's lymphoma</td>
<td>17</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Leukemias</td>
<td>16</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Solid tumors</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Other diseases</td>
<td>32</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Adults</td>
<td>Leukemias</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Carcinomas</td>
<td>15</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>33</td>
<td>26</td>
<td>2</td>
</tr>
</tbody>
</table>

Frequent plasmapheresis is known to maintain high levels of viral antibodies (21), this γ-globulin conjugate was suitable for staining of mumps as well as several other viral antigens (see below), and elicited brilliant fluorescence also in a fraction of the EB cells (Fig. 1C and D). The reaction was blocked by prior exposure of the fixed cell smears to human sera which gave positive reactions in the indirect test, but not by negative sera (Table 5). Sera from rhesus monkeys, rabbits,
guinea pigs, calves, and horses failed to block staining by the human \( \gamma \)-globulin conjugates. The labeled mumps hyperimmune \( \gamma \)-globulin was employed predominantly in further analysis of the staining reaction.

Relation of degenerating ana dead cells to the staining reaction. As pointed out earlier, the immunofluorescent cells often appeared to be in a state of degeneration, and therefore the possibility had to be ruled out that dead cells present in the cultures adsorbed the labeled proteins nonspecifically. Although the percentage of fluorescent cells was generally smaller than that of dead cells as determined by staining with trypan blue, a more definitive analysis was needed. Consequently EB cells were subjected to various procedures which increase their death rates: (i) reduction of the serum concentration in the media to 1% or nil; (ii) crowding of the cells during cultivation in the tips of pointed centrifuge tubes; and (iii) incubation of the cultures without refeeding for up to 2 weeks. Even though such preparations contained from 30 to 80% trypan blue-staining cells, immunofluorescence was elicited only in the usual low percentage of the cells.

In several experiments, cells were stained vitaly; i.e., without prior fixation. The results were similar to those obtained by the routine technique which are shown in Fig. 1. The stained cells again showed, as a rule, evidence of degeneration. It was impossible, therefore, to decide whether this fluorescence was due to surface staining or to penetration of the labeled proteins into the degenerating cells. In addition, the faint stippling of some of the cells, noted on occasion after staining of fixed preparation (Fig. 1A) was somewhat more pronounced on vital staining, especially by the indirect technique. Indeed, the stippling (but not the intense staining of the small proportion of the cells) was seen after vital staining solely by the anti-human globulin conjugate. It clearly outlined the circumference of the cells and seemed to cover also the cell surface.

Comparison of various cell lines. The five Burkitt lymphoma cell lines as well as various other cells were compared in direct as well as indirect immunofluorescence tests. The results are summarized in Table 6. In the direct tests, all Burkitt lymphoma lines contained a variable proportion of cells which stained with the labeled human mumps hyperimmune and normal \( \gamma \)-globulins. The percentage of staining cells was highest in EB3 preparations, followed in decreasing order by the EB1, EB2, SL1, and AL1 cells. This order appears to match the reported ease with which virus particles have been detected in sectioned cells under the electron microscope (5, 8; Epstein et al., in press). The AL1 line originally contained sufficient virus particles to be readily found, but by the time the line was received none was detected (Rabson et al., in press). Two of the other lines yielded positive immunofluorescence in a very low percentage of the cells; i.e., the SKL-1 and SKL-2 lines which were isolated from American patients with leukemias (Clarkson, to be published). It has not been reported whether these cultures contain virus particles. The staining was blocked by prior exposure of the SKL cells to human sera which gave positive results in the indirect tests with EB cells, but not by negative sera (Table 5). The 64-10 line, which at one time harbored a virus (14), and the LK-1D
line gave consistently negative results, and so did numerous cultures of normal peripheral leukocytes, stimulated by phytohemagglutinin. The last type of cultures was examined within 1 to 16 days of incubation. Various routine cell cultures (HeLa, WI-38, HEK, primary rabbit kidney, and Earle's strain L) likewise failed to reveal positive cells.

The results of direct staining were matched by those obtained with the indirect technique, with the use of a positive serum for the primary phase of the reaction. The controls, omitting the primary serum, revealed that the rabbit anti-human globulin conjugate alone elicited fluorescence in some of the cell lines. As mentioned earlier, the three types of EB cells showed at most a fine stippling of moderate intensity under these conditions, especially on vital staining. Similar results were obtained with the SL1 and the AL1 lines. The stippling appeared to be a surface reaction. In contrast, many of the 64-10, SKL-1, SKL-2, and LK-1D cells, and a few of the cells in peripheral leukocyte cultures, revealed granular cytoplasmic staining of moderate to marked intensity when exposed to rabbit anti-human globulin conjugates. Rabbit anti-guinea pig globulin conjugates gave very weak staining, a not unexpected cross-reaction (13). The per-

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**Table 5. Blocking by various sera of direct staining of EB3, SKL-1, and SKL-2 cells by human mumps hyperimmune γ-globulin conjugate**

<table>
<thead>
<tr>
<th>Blocking serum</th>
<th>Species</th>
<th>No.</th>
<th>Indirect staining</th>
<th>Staining by labeled human mumps hyperimmune γ-globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>B-2239</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B-1145</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B-1819</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PK</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LK-6</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LK-15</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LK-33</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LK-43</td>
<td>++</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>3*</td>
<td>NT†</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>5</td>
<td>NT</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>4</td>
<td>NT</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>4</td>
<td>NT</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Calf</td>
<td>4</td>
<td>NT</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Number of animal sera tested.
† Not tested.

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**Table 6. Direct and indirect staining of various cell lines**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Direct test (a)</th>
<th>Indirect test (b)</th>
<th>Control of indirect test (c)</th>
<th>Per cent of cells positive (a or b), range</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1-3</td>
</tr>
<tr>
<td>EB2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0.5-2</td>
</tr>
<tr>
<td>EB3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>5-10</td>
</tr>
<tr>
<td>SL1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ca. 1</td>
</tr>
<tr>
<td>AL1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ca. 0.1</td>
</tr>
<tr>
<td>64-10</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LK-1D</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ca. 10</td>
</tr>
<tr>
<td>SKL-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.1-0.5</td>
</tr>
<tr>
<td>SKL-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.1-0.5</td>
</tr>
</tbody>
</table>

* Positive human serum plus FITC-conjugated rabbit antibodies to human globulin.
† Only FITC-conjugated rabbit antibodies to human globulin.
* Except for occasional fine surface stippling of some of the lymphoma cells.
4 Not done.

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**Table 7. Staining of various viral antigens by FITC-conjugated human mumps hyperimmune γ-globulin**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mumps</td>
<td>HeLa</td>
<td>++</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>HEK</td>
<td>++</td>
</tr>
<tr>
<td>Varicella</td>
<td>HEK</td>
<td>(±)</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>WI-38</td>
<td>-</td>
</tr>
<tr>
<td>Reo</td>
<td>Type 1</td>
<td>FL</td>
</tr>
<tr>
<td></td>
<td>Type 2</td>
<td>FL</td>
</tr>
<tr>
<td></td>
<td>Type 3</td>
<td>FL</td>
</tr>
<tr>
<td>Simian virus 40</td>
<td>HEK</td>
<td>-</td>
</tr>
<tr>
<td>Influenza A</td>
<td>HeLa</td>
<td>+ (S and V antigen)</td>
</tr>
</tbody>
</table>
percentage of stained cells in the four lines mentioned varied to some extent from test to test. The nature of this type of reaction is still obscure, especially since it is blocked significantly, if not totally, by prior exposure of the cells to human sera or globulin fractions.

Attempts to relate the immunofluorescence of lymphoma cells to viral antigens. For the indirect method of staining of EB cells, sera were used which had been collected from patients infected with herpes simplex, varicella or herpes zoster, and cytomegalic inclusion disease viruses, as well as from individuals with known antibody levels to these agents, as determined by complement fixation or virus-specific immunofluorescence. In each category, only a proportion of the sera gave positive results in line with the expected percentages for given age ranges.

The labeled mumps hyperimmune γ-globulin was tested against a number of viral antigens (Table 7). It stained herpes simplex antigen(s) brilliantly, whether present in human embryonic or rabbit kidney cells. The conjugates failed to stain varicella virus-infected HEK cells, except for a barely detectable dull-green coating, insufficient to account for the brilliant fluorescence elicited in the lymphoma cells. No staining was obtained of appropriate cells infected by cytomegalovirus (Davis strain), simian virus 40, or reovirus types 1, 2, and 3. In addition, attempts were made to stain lymphoma and some of the other cell lines with FITC-conjugated rabbit antibodies to herpes simplex virus or the reoviruses with negative results. The tests concerning reovirus were kindly performed by R. Spendlove, Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley. In addition, no specific staining was noted with labeled monkey antibodies versus murine leukemia (Rauscher) virus (11).

None of the above tests appeared to link any of the viruses studied to the immunofluorescence elicited in the lymphoma cells. Nevertheless, since the FITC-conjugated human γ-globulins stained herpes simplex antigens, additional tests were carried out for a final exclusion of this agent. The mumps hyperimmune conjugate was absorbed with concentrated herpes simplex virus grown either in rabbit or human embryonic kidney cultures. Volumes of 1 ml of the conjugate were mixed with an equal volume of either centrifugally concentrated herpes simplex virus or Hanks’ solution, incubated at 37°C for 1 hr and overnight at 4°C, and centrifuged thereafter at 100,000 × g for 1 hr. The supernatant fluids, representing a twofold dilution of the original conjugate, were used for staining of EB1, EB3, herpes simplex virus-infected and normal HEK or rabbit kidney cells. The results (Table 8) showed that the absorbed conjugates no longer stained HSV antigens, but the reaction with the expected proportion of EB cells was unaffected.

**DISCUSSION**

The immunofluorescence obtained with the various cell lines tested appears to fall into at least three categories.

The first type, the brilliant fluorescence observed in a low percentage of the lymphoma cells (Fig. 1), was obtained by the indirect method only with certain human sera and anti-human γ-globulin conjugates. Direct staining by FITC-coupled pooled human γ-globulins was blocked by human sera which gave positive reactions in the indirect test but not by negative human or animal sera. It would seem that this type of reaction is due to specific antibodies to an as yet unidentified antigen(s).

The second type, the cytoplasmic fluorescence of moderate to marked intensity elicited by FITC-conjugated rabbit antibodies to human globulins only in cells of the lines derived from various leukemias and of normal leukocyte cultures, has not been analyzed, and its nature is at present obscure. Aside from the fact that the percentage of staining cells in given lines was variable from test to test, this type of staining was unexplainably reduced or totally blocked by prior exposure of the cells to human sera or globulin, and therefore is assumed to be due to nonspecific factors.

The third type of fluorescence likewise has not been clarified; i.e., the fine surface stippling of moderate intensity (Fig.1A) observed occasionally on vital staining of lymphoma cells in the indirect tests with human sera, or in direct tests with human γ-globulin or rabbit anti-human globulin conjugates. It might be due to

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**Table 8. Absorption of FITC-conjugated human mumps hyperimmune γ-globulin by herpes simplex virus (HSV)**

<table>
<thead>
<tr>
<th>FITC-conjugate</th>
<th>Absorbent</th>
<th>EB1</th>
<th>EB3</th>
<th>HSV infected</th>
<th>HEK infected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human mumps hyperimmune</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HSV (rabbit kidney)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HSV (HEK)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit anti-HSV</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
nonspecific adsorption of labeled proteins or to isoantibodies. In any event, it is readily differentiated from the first type of staining reaction which has been the main subject of this report.

It is tempting to ascribe the brilliant immunofluorescence elicited in a small proportion of the lymphoma cells to the virus carried by a fraction of the cells in these cultures. Indeed, several observations support this possibility. (i) There seems to be a rough correlation between the percentage of stainable cells in the five cell lines derived from Burkitt's lymphoma and the percentage of cells which harbor virus particles as determined by electron microscopy. Although the electron microscopic studies do not lend themselves readily to accurate quantitation, about 1 to 2% of the EBI and EB2 cells were estimated to harbor virus particles (5, 8; Epstein et al., in press). The percentage of immunofluorescent cells in these two lines has fluctuated in numerous tests between 1 and 3 and between 0.5 and 2%, respectively. In the EB3 line, which contains more virus-infected cells than the first two (Epstein et al., in press), the stainable cells have ranged between 3 and 10%. The percentage of stainable cells in the AL1 line was in the order of 0.1 when cells containing virus particles could no longer be found (Rabson et al., in press). (ii) The cells revealing virus particles, especially when found in the cytoplasm, exhibited marked degrees of degeneration (10), as did the cells showing immunofluorescence. Finally (iii), exposure of EBI and EB3 cultures to 5-methylamino-2'-deoxyuridine (MADU), kindly supplied by M. R. Hilleman, Merck Sharp & Dohme, Inc., West point, Pa., reduced significantly the percentage of stainable cells within 7 to 10 days (Henle and Henle, to be published). MADU was shown recently to be of low toxicity for cultured cells and effective in the treatment of cultures infected with herpes simplex virus (15). These points provide only indirect evidence, and final proof of the identity of the stainable and virus-carrying cells must still be furnished.

If the above interpretation should turn out to be correct, the virus present in the lymphoma cultures would not be restricted to the regions of prevalence of Burkitt's tumor but would be widespread also in the United States on the basis of the indirect immunofluorescence test. The increase in the incidence of reactive American sera from about 30% in children to more than 90% with advancing age of the serum donors resembles the gradual acquisition by populations of antibodies to common viruses. The significance of the fact that positive immunofluorescence was obtained with all of 17 sera from African children suffering from Burkitt's lymphoma cannot be assessed because no sera have been available from other African children for comparison.

Needless to say, it is still unknown whether the virus present in the lymphoma cell cultures is etiologically related to Burkitt's tumor or represents merely a passenger. The finding of the same kind of virus particles in five cell lines isolated from four patients in three different laboratories tends to favor the first alternative, but, obviously, further cell lines need to be isolated and examined for the presence of virus particles and immunofluorescence. The wide distribution of antibodies, presumably to this virus, would not necessarily preclude an etiological relationship, since some oncogenic animal viruses are widespread among their respective hosts, evoking immune responses but rarely tumor formation. Furthermore, cases resembling Burkitt's lymphoma have been reported recently to occur in the United States (4, 17). Finally, two cell lines isolated from American leukemic patients (SKL-1 and SKL-2) have revealed a very small percentage of cells, which were stainable by the same procedures as employed for the lymphoma cultures. As in the case of EB cells, staining by FITC-conjugated human γ-globulin was blocked by prior exposure of the SKL cells to sera from patients with Burkitt's lymphoma and other sera which gave positive reactions in the indirect immunofluorescence tests, but not by negative sera. The presence of cell-associated virus particles in these two lines has not been reported. They would be difficult to find under the electronmicroscope if the assumption is correct that mainly the few staining cells harbor the agent.

All attempts to relate the staining to known viral antigens have failed. Morphologically, the virus resembles the herpes group, and consequently efforts were concentrated upon herpes simplex, varicella, and cytomegaloviruses. In addition, reovirus was considered merely because of the reported isolation of a member of this group from a Burkitt tumor (2). All these agents seem to be excluded by the tests recorded. Only a proportion of sera from patients infected with these agents or from individuals with known antibody levels to them elicited fluorescence in indirect tests with EB cells. The FITC-conjugated human γ-globulins failed to detect varicella virus, cytomegalovirus (Davis strain), or reovirus types 1, 2, and 3 antigens in appropriate, infected host cells. Although these conjugates stained herpes simplex antigen(s) brilliantly, this reactivity could be removed completely by adsorption with herpes simplex virus without affecting the staining of EB cells. Efforts to elicit fluorescence in the lymphoma cells by
labeled rabbit antibodies to herpes simplex virus
or the three types of reoviruses, likewise, gave negative results. It would seem that the cells
carry an as yet unidentifiable virus which re-
sembles in size and shape members of the herpes
group.

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Addendum in Proof
Four additional Burkitt lymphoma cell lines, iso-
lated by Drs. Pulvertaft and Osunkoya, were supplied
by Dr. R. Manaker of the National Cancer Institute
and subjected to direct and indirect immuno-fluo-
rescence tests without knowledge of the results of electron
microscopic examinations. Two of these (P 2002 and
P 2003) revealed from 1–2 and about 5% staining
cells, respectively; the other two, designated P 2004
and P 2005 (Reji), were negative. These results again
responded roughly to the number of cells shown to
contain virus particles. The two negative cell lines
had been in culture for more than 3 years; the positive
lines were 10 and 3 months old.

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