Prevalence of Precipitating Antibody to Antigens Derived from Burkitt Lymphoma Cultures Infected with Herpes-Type Virus (EB Virus)

By David A. Stevens, Thomas W. Pry and Elizabeth A. Blackham

Cells derived from Burkitt's lymphoma have been successfully cultured and have been found to contain an apparently hitherto undiscovered member of the herpesvirus group (herpes-type virus–HTV or Epstein–Barr virus–EBV). Since this time, considerable interest has centered on seroepidemiologic surveys of various disease categories and healthy populations for antibodies against antigens associated with these infected cells. Serologic methods used have included indirect immunofluorescence, immunodiffusion, complement fixation, and antibody coating. This interest has been heightened by the finding of similar, if not identical, viruses in some cell lines derived from Burkitt's lymphoma of non-African origin, lymph nodes from patients with other cancers, and from marrow and/or peripheral blood of patients with acute or chronic leukemias, infectious mononucleosis, and normal individuals. This agent also has been implicated as possibly etiologic in infectious mononucleosis and Burkitt's lymphoma.

The present study reports our experience in developing a sensitive assay for precipitating antibody in human sera, results with 517 sera, and related studies. The evidence that the precipitins are virus-specific, and the correlation with other serological procedures are discussed.

Materials and Methods

Microscale Ouchterlony immunodiffusion technique. The Ouchterlony method employed in these studies uses a plastic template embedded on a thin layer of agar on a microscope slide. Following diffusion for 48–72 hours, slides are treated with a cadmium buffer as a developer. The enhanced sensitivity of the template method compared to other Ouchterlony techniques has been emphasized in recent comparative studies.

Soluble cell-derived antigen. Studies of soluble antigens derived from lymphoma cell lines were initiated as part of a project to compare immunologically herpes-type viruses from cell lines of different origins, and optimal test sensitivity was desired. Efforts to achieve this included the following: use of a cell line with increased virus content, and manipulation to increase virus yield and to increase the sensitivity of the microdiffusion method. The HR1K clone of the P3J line was selected because it contained more virus as judged by indirect immunofluorescence. Aging of cultures has also been shown to increase the immunofluorescence reacting antigen. Therefore, cultures used to prepare immunodiffusion antigen were aged to the point of peak reaction by this test as detailed below.

Preparation of soluble antigen, as modified from the method of Old et al., was as

From the Viral Biology Branch, National Cancer Institute, Bethesda, Md.
First submitted February 14, 1969; accepted for publication August 27, 1969.

David A. Stevens, M.D.; Thomas W. Pry, M.S.; Elizabeth A. Blackham, M.T.; Microbiology Section, Viral Biology Branch, National Cancer Institute, Bethesda, Md.
Reprint requests to: Dr. David A. Stevens, Dept. of Medicine, U.C.L.A. Medical Center, Los Angeles, Calif. 90024.
follows: Cells were maintained at 37°C in Eagle's minimal essential medium with glutamine and 20–30 per cent fetal calf serum in 32 oz. prescription bottles with an air: fluid ratio of 7:1. Feeding schedule of stock cultures was adjusted according to cell counts and immunofluorescence to allow optimal virus content. In practice this meant that the cell concentration was allowed to vary between 5–15 × 10⁶ cells/ml, which required feeding approximately twice per week. Cultures were periodically examined for bacteria, fungi, and Mycoplasma.

When sufficient volume was attained, cells were seeded in 1050 ml. of culture at a concentration of 1 × 10⁶/ml. These bottles were placed at 33°C for 13 days without further feeding, resulting in more than 50 per cent cell death as determined by the trypan blue exclusion test. The harvest was pelleted by slow centrifugation, resuspended in buffered saline, frozen and thawed, and pulverized in a glass tissue grinder until all visible particulate matter was homogenized.

This material was sonicated in three 30-second intervals at 6-μ amplitude, and clarified by slow centrifugation. After further centrifugation at 20,000 r.p.m. for one hour, the supernatant fluid was concentrated by dialysis in the cold, overnight, against a 50:50 mixture of Aquacide I and II (Calbiochem). It was then dialyzed again in the cold against phosphate-buffered saline for four hours. The final volume was adjusted to 3.3 ml. by the addition of buffered saline. This material had a protein concentration of 1.5 gm. per cent (Folin–Ciocalteu reagent).

Testing procedure. Tests were routinely run in duplicate, and usually in quadruplicate. A positive control serum, usually a serum from a Burkitt's lymphoma patient giving three–four precipitin lines against the purified virus antigen, was used in every test. This made it possible to refer the results on all test sera back to the P3J virus–Burkitt patient system. When a large pool of strongly reacting normal donor serum became available, this was also used as a reference serum. Positive sera were tested against fetal calf serum as antigen to rule out reactions against this component of the medium.

Serum specimens. Sera were obtained from numerous sources. These included 119 sera from 73 African patients with Burkitt's lymphoma. Individual sera from healthy Africans, relatives of Burkitt lymphoma patients, Africans with other solid tumors and African patients with "other pathology" (all pathology except malignancy and infectious mononucleosis) collected from the same hospitals as the sera from Burkitt lymphoma patients were tested as a control group. Sera from healthy North Americans and Americans with various diseases were also tested.

These groups could be divided into subgroups principally comprised of either adults (over 15 years of age) or of children (under 15 years). For example, of 88 Burkitt lymphoma serum specimens for which the age was given, 85 were collected from patients under 15 years of age; 13 of 14 acute lymphocytic leukemia patients for which the age was given were under 15 years. Sera were available from 120 American children as a control for these groups.

Specimens available from acute myelocytic leukemia patients came principally from adults (15 of 17 sera). Serial sera were available in some instances from infectious mononucleosis patients (42 sera from 28 patients), all university students. Twenty-seven pools of adult human donor sera were purchased commercially from the Interstate Blood Bank, Tennessee. Sera from 141 healthy American adults served as a control.

Of 36 African control sera for which the age was known, 22 were from children under 15 years; of 29 Africans, excluding those with tumors, 16 were in this age group.

Electron microscopic studies. The cell-derived soluble antigen was examined by negative staining for presence of virus particles. Individual precipitin bands cut from the agar with a scalpel were similarly examined by thin section. Electron microscopic studies were kindly performed by Dr. Albert J. Dalton and Dr. Victor H. Zeve, of the National Cancer Institute.

Trypsinization. Three-tenths milliliters of cell-derived antigen was treated with 0.2 ml. of 0.5 per cent trypsin for one hour at 37°C. A similar aliquot was treated with medium without trypsin as a control, and the products were tested against reference sera.

Absorption of sera. Equal amounts of either cell-derived antigens or centrifuged cell
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Fig. 1.—Identity of reactions in sera of six healthy American blood donors (peripheral wells) and cell-derived antigen (center well).

pellets and serum were incubated one hour at 37°C with agitation every 15 minutes. The mixtures were then incubated overnight at 4°C, centrifuged, and the supernatant fluids tested in immunodiffusion.

Heterologous antisera. Antisera in rabbits were prepared by Dr. S. A. Mayyasi, Chas. Pfizer and Co., Maywood, N. J., by initial foot-pad inoculation of virus in Freund's adjuvant followed by four weekly intramuscular injections of virus. Aliquots of antisera prepared in monkeys and guinea pigs by the intraperitoneal inoculation of gradient purified HTV in complete Freund's adjuvant, followed by a subcutaneous booster inoculation, were kindly provided by Dr. Mary A. Fink, National Cancer Institute.

Reference antisera to other agents. Antisera to other viruses and to Mycoplasma were obtained from the National Institute of Allergy and Infectious Diseases, Research Reference Reagents Branch. Method of preparation and immunological activity of these sera are described elsewhere. Sheep antisera to Candida albicans and to Candida tropicalis containing precipitating antibodies were kindly supplied by Dr. H. Hasenclever, National Institute of Allergy and Infectious Diseases.

RESULTS

The modifications of the micro-Ouchterlony technique for the study of soluble antigens from HTV-infected cells described above were felt to increase sensitivity of the immunodiffusion test, with increased clarity and number of precipitin bands, as compared to the results in our hands with the same reference sera using the previously described immunodiffusion methods.

Reactions of various sera. Sera from the various groups detailed below which reacted with the antigen all gave one strong band ("A") in common (Fig. 1). Sera giving two precipitin lines also had a second strong band ("B") in common, closer to the antibody well. Occasional sera gave a fainter third precipitin reaction of identity with reference sera ("C"), which appeared between B and the antibody well. These precipitins appear to be virus specific, as determined by absorption studies described below.

In addition to these precipitins, some sera demonstrated a variably positioned precipitin band against purified virus or cell-derived antigens which gave a reaction of identity with an antigen in fetal calf serum. This occurred
Fig. 2.—Peripheral wells A–F: sera of leukemic patients. Center well: cell-derived HR1K antigen. Serum E, from transfused patient, demonstrates precipitating antibody of broad specificity (continuous band with antigen and adjacent serum wells). Fine precipitin reactions demonstrated by sera A, C, D and E (fine line close to serum well) could be shown to give reaction of identity with positive control serum from Burkitt lymphoma patient.

with less than five per cent of normal adults or children from the United States, but was not seen with any sera from Africans which we tested. The rarity of this precipitating system is in agreement with previous reports. Any difficulty in interpretation was minimized by use of a positive control serum.

Occasional sera gave unusual broad bands against only the cell-derived antigen from the HR1K cell line, with antigen similarly prepared from one or two similar cell lines (NC37 and RPMI 3306) which were free of virus by immunofluorescence and electron microscopy, and sometimes with all of these as well as with the sera in the adjacent wells. These did not resemble nor give reactions of identity with the precipitin lines obtained with positive control reference sera (Figs. 2, 3). These precipitin reactions could generally be related to a recent history of blood transfusion, and accordingly were seen almost exclusively in the serum of leukemic patients.

Table 1 shows the number of precipitin reactions of identity with positive reference sera for the groups tested. As can be seen, the African group with Burkitt's lymphoma shows interesting differences from African and American children by this serological test. For example, a comparison of African Burkitt
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Fig. 3.—Top well: cell-derived antigen from HTV-containing cell line (HR1K). Bottom well: antigen prepared by identical method from HTV-free cell line. Right well: serum of American patient who had previously received blood transfusions. Broad precipitin reaction with HTV-free cell line is characteristic of that found in other transfused patients, and presumably represents isoantibody. No identity with fine precipitin reaction with HR1K antigen.

lymphoma patients (Table 1, Group I) with African controls (Group II) by the Student-Fisher t test for mean number of bands and per cent positive would show p values of < 0.001. The application of these same tests to the comparison between Burkitt patients and American children (Group III, 3) gave p values of < 0.001. However, the validity of such comparisons is dependent on the sampling methods used, as discussed below. Also, there may be differences in precipitating antibody between African non-Burkitt and American populations. The samples from other groups in Table 1 are small. In addition, the only sera we have tested to date which give four precipitin lines have all been from patients with Burkitt's lymphoma, with one exception. This latter serum was from a 50-year-old Ghanian man with a diagnosis of "lymphoid hyperplasia." No further clinical information or followup sera are available for this case.

Table 2 is presented as a summary of this information, showing the mean number of bands, per cent positive, and the per cent with multiple bands in each patient category tested. These categories were grouped into childhood and adult groups (see Materials and Methods) for comparison.

Table 3 presents the results with the American normal children and children with "other pathology," tabulated by age. There are no significant differences in these childhood age groups, and it appears that many children acquire antibody at a very early age—certainly by age two. Adequate numbers
Table 1.—Relationship of Number of Precipitin Bands and Diagnosis

<table>
<thead>
<tr>
<th>Number of Precipitin Bands</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. African—Burkitt's lymphoma</td>
<td>5 †</td>
<td>8</td>
<td>11</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1. Uganda (34) *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Ghana (59)</td>
<td>4</td>
<td>22</td>
<td>16</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>3. Nigeria (26)</td>
<td>1</td>
<td>18</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total Burkitt's lymphoma (119)</td>
<td>10</td>
<td>48</td>
<td>31</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>II. African controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Ghana—normal and “other pathology” †</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2. Uganda—normal and “other pathology”</td>
<td>6</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total African normal and “other pathology” (30)</td>
<td></td>
<td></td>
<td>14</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3. Ghana—other malignancy</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total African controls (37)</td>
<td>13</td>
<td>18</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>III. American controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Pooled normal adult donor sera</td>
<td>3</td>
<td>23</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. American adult healthy individuals (141)</td>
<td>57</td>
<td>59</td>
<td>22</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3. American healthy children (120)</td>
<td>83</td>
<td>34</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total American controls (261)</td>
<td>140</td>
<td>93</td>
<td>25</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>IV. Americans—hematologic diseases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Infectious mononucleosis (42)</td>
<td>15</td>
<td>20</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2. Acute lymphocytic leukemia (14)</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Acute myelocytic leukemia (17)</td>
<td>6</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Total number of sera tested in parentheses.
† Number of individual sera demonstrating number of specific precipitin bands at head of column.
‡ Miscellaneous diseases, except malignancy and infectious mononucleosis.

Table 2.—Summary of Immunodiffusion Data

<table>
<thead>
<tr>
<th>Mean Number of Bands</th>
<th>Percent Two or More Precipitin Bands</th>
<th>Percent Three or More Precipitin Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Pediatric groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Burkitt lymphoma sera (119) *</td>
<td>1.74</td>
<td>92</td>
</tr>
<tr>
<td>1b. Burkitt lymphoma patients (73) †</td>
<td>1.73</td>
<td>92</td>
</tr>
<tr>
<td>2. American healthy individuals (120)</td>
<td>0.33</td>
<td>31</td>
</tr>
<tr>
<td>3. Acute lymphocytic leukemia (14)</td>
<td>0.50</td>
<td>43</td>
</tr>
<tr>
<td>II. Adult groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. American healthy individuals (141)</td>
<td>0.79</td>
<td>60</td>
</tr>
<tr>
<td>2. Acute myelocytic leukemia (17)</td>
<td>0.71</td>
<td>65</td>
</tr>
<tr>
<td>3a. Infectious mononucleosis sera (42) †</td>
<td>0.84</td>
<td>64</td>
</tr>
<tr>
<td>3b. Infectious mononucleosis patients (28) †</td>
<td>0.75</td>
<td>61</td>
</tr>
<tr>
<td>III. Inclusive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. African healthy and “other pathology” (30)</td>
<td>0.87</td>
<td>63</td>
</tr>
<tr>
<td>2. American controls (261)</td>
<td>0.58</td>
<td>46</td>
</tr>
<tr>
<td>3. All acute leukemia (31)</td>
<td>0.61</td>
<td>55</td>
</tr>
</tbody>
</table>

* Total sera tested in parentheses.
† Serial sera were available from some patients, and the data is also summarized using only the first sample available from each patient.
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Table 3.—Precipitating Antibody in American Children, by Age

<table>
<thead>
<tr>
<th>Age</th>
<th>Number Positive/Total Sera</th>
<th>Per cent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 months to &lt; 2 years</td>
<td>8/20</td>
<td>40</td>
</tr>
<tr>
<td>2 years to &lt; 4 years</td>
<td>4/20</td>
<td>20</td>
</tr>
<tr>
<td>4 years to &lt; 6 years</td>
<td>5/20</td>
<td>25</td>
</tr>
<tr>
<td>6 years to &lt; 8 years</td>
<td>8/20</td>
<td>40</td>
</tr>
<tr>
<td>8 years to &lt; 10 years</td>
<td>6/20</td>
<td>30</td>
</tr>
<tr>
<td>10 years to &lt; 12 years</td>
<td>6/20</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.—Correlation of Precipitin Bands and Fluorescent Antibody Titer

<table>
<thead>
<tr>
<th>Fluorescent Antibody Titer (Reciprocal of Highest Dilution Showing Positive Immunofluorescence)</th>
<th>Number of Precipitin bands</th>
<th>&lt; 10</th>
<th>10</th>
<th>40</th>
<th>160</th>
<th>640</th>
<th>2560</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11 *</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>21</td>
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<td>0</td>
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<td>3</td>
<td>9</td>
<td>5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>2</td>
<td>6</td>
<td>13</td>
<td>16</td>
<td>8</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

* Number of individual sera demonstrating number of specific precipitin bands at left of row and fluorescent antibody titer at top of column.

of sera were not available to compare age of onset of antibody between geographic regions or socioeconomic groups.

There was no relation between precipitin reaction and the presence or titer of heterophile antibody in infectious mononucleosis patients sampled from the early acute through convalescent phases of the disease. In instances where serial sera were available, precipitating antibody persisted after heterophile antibody had subsided. Several heterophile-positive precipitin-positive infectious mononucleosis sera which were absorbed with beef erythrocytes and guinea pig kidney cells sufficiently to remove hemagglutinating activity for sheep erythrocytes retained precipitating activity.

Where serial samples were available the gel diffusion results were identical with few exceptions. These exceptions possibly could be explained by variations in clinical status or therapy, or by technical variations. There was insufficient clinical information available to attempt correlation in these few instances with stage of disease or therapy.

Other microdiffusion studies

Comparison of precipitin bands with fluorescent antibody titer. Fifty-six sera from normal individuals and patients with various diseases were kindly supplied by Dr. L. J. Old and Dr. Y. Hirshaut, Sloan-Kettering Institute. These were tested by Dr. Hirshaut by the indirect immunofluorescent test, and in this laboratory using cell-derived antigen in immunodiffusion. The results are given in Table 4. The rank correlation coefficient for these two tests is + 0.79, demonstrating that titer of immunofluorescent antibody corresponds closely to number of precipitin bands, with the former test the more sensitive in these
two laboratories. All statistical analyses were performed by Dr. Deward Waggoner, National Cancer Institute. Similar results were obtained with other groups of sera tested by both indirect immunofluorescence as performed in three other laboratories and immunodiffusion.

**Relationship to other agents.** Rabbit and mouse antisera to *Herpesvirus hominis* (courtesy of Dr. Charles Daniels, National Institute of Dental Research) and reference guinea pig antisera to *Herpesvirus hominis*, *Herpesvirus simiae* B, and *Herpesvirus suis* were without precipitating activity. Since sera from infectious mononucleosis patients have been shown to have precipitating antibody to strains of Newcastle Disease Virus, guinea pig antiserum to the Roakin strain of this virus giving discrete precipitin bands against the homologous virus was also tested and found negative.

Similarly, reference antisera to common laboratory contaminants, i.e., *Mycoplasma pneumoniae*, *Mycoplasma salivarium*, *Mycoplasma orale*, *Mycoplasma hominis*, *Candida albicans* and *Candida tropicalis* did not show precipitating antibody to HR1K-derived antigen.

Moreover, selected human sera with known high-neutralizing or complement-fixing antibody titer to *herpes simplex* did not have precipitating antibody to HR1K antigen, and the antisera to purified P3J virus prepared in rabbits could not be shown to either neutralize *herpes simplex* or sensitize it for further neutralization by goat antirabbit globulin serum (Dr. Charles Daniels, personal communication).

**Heterologous sera and absorptions.** Various lots of antisera prepared as described in heterologous species demonstrated four to seven precipitin bands with HR1K cell-derived antigens. Following absorption with fetal calf serum, sheep erythrocytes, human type-A and -B erythrocytes, and with five human monolayer tissue-culture cell lines (HeLa, WISH, KB, Chang, HEp-2) which contain all known human leukocyte antigens, some sera then gave precipitin bands which showed reactions of identity with A, or A and B, when tested in parallel with human reference sera.

Repeated absorption of reference human sera with untreated centrifuged cell pellets of HTV-free cell lines, the same cell pellets treated with acetone to expose internal antigens, or the cell-derived soluble antigens prepared by the method described from HTV-free cell lines failed to remove precipitating activity, while absorption with the cell-derived soluble antigens from HTV-containing cell lines completely removed the precipitating activity.

**Trypsinization.** Two reference sera which gave four precipitin bands with cell-derived antigen showed no activity against the trypsin-treated antigen and three bands against the treatment control antigen. The obliteration of all antigenic activity with trypsin treatment suggests the antigens are protein in nature; the loss of one weak precipitin band following the control treatment of the antigen is probably due to dilution.

**Electron microscopic studies.** Negative staining of the cell-derived antigen and thin sectioning of precipitin bands cut from the agar failed to reveal any virus structures.

**Identity of purified virus antigen and soluble cell-derived antigen.** Batches
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Fig. 4.—Identity of purified virus antigen (bottom well) and cell-derived antigen (top well). Right well contains serum giving two precipitin bands with each antigen.

of gradient purified HTV containing high virus particle counts prepared from the P3F cell line and similar to those employed in previously reported immunodiffusion studies were provided by Mr. I. Toplin, Chas. Pfizer and Co. Pairs of selected reference sera giving zero, one, two, three and four precipitin bands with HR1K cell-derived soluble material were used for comparison with the gradient-purified virus antigen. With selected batches of virus antigen, these sera were either both negative or produced the same number of bands with both antigens. Reactions of identity of A and B precipitins could be demonstrated with ease (Fig. 4), and the C precipitin on repeated testing. A fourth band, as appeared with some Burkitt lymphoma patients' sera, could be seen with both antigens but was too faint to merge and clearly determine identity or nonidentity.

Titration. Attempts to quantitate the technique further by serial dilution of serum or antigen met with limited success, as is common with many immunodiffusion systems. Our strongest sera giving multiple, clear bands could be diluted to 1:4–1:8 before losing activity. With such sera used undiluted, the antigen could be diluted to 1:8–1:16 before reactions became unreadable.

DISCUSSION

This study documents the high incidence of precipitating antibody to antigens derived from HTV-infected lymphoma cell lines. The evidence at hand suggests that the precipitin reactions described are virus-specific, rather than due to isoantigens or other antigens.

Several findings provided evidence against the latter possibility. Tissue-reactive isoantibodies, as could be induced by transfusion, could be detected by the use of reference sera (as could antibodies to components of the medium in which these cell lines are grown). While precipitating antibody in selected
human sera to DNA, soluble and particulate nucleoprotein,\(^{25}\) and to other antigenic systems such as Sm\(^{25}\) and Australia antigens has been described.\(^{26}\) The high incidence of activity in sera tested from normal individuals, plus the absence of identical reactions with antigen from HTV-free cell lines provides evidence that these antigen-antibody systems are not responsible for our results. There was also no correlation of precipitin reaction with ABO or Rh blood group.

Data from other studies\(^{17a,b}\) comparing cell-derived antigens prepared as described from virus-free (by immunofluorescence and electron microscopy) and virus-containing lymphoblastoid cell lines from different tissues and disease states from human and simian sources, and from primary lymphoid and hemic tissue specimens, demonstrate that identical soluble antigens can be prepared from virus-containing human cell lines only. The virus-containing cell lines which demonstrated these antigens include, in addition to HR1K, the F152, F132, and EB3 cell lines of hemic origin. In studies\(^{6}\) using gradient purified virus as antigen it was noted that (a) only the density gradient cut containing the virus and (b) only virus gradient cuts from different runs which contained \(> 1.5 \times 10^{10}\) particles/ml possessed antigenic activity, regardless of protein content, and (c) purified virus from several HTV-containing cell lines was antigenically identical, while cuts at the same gradient density from many virus-free cell lines were free of antigenic activity. These findings provide evidence of virus specificity of the precipitin reactions.

Absorption studies further suggest the specificity of these reactions. After absorption of heterologous sera with medium components and human antigens (such that antigen excess could be demonstrated in the sera) reactions of identity with human reference sera remained. Absorption of human sera with centrifuged human cell pellets or soluble antigens prepared from these cells failed to remove precipitating antibody except when antigen from the HTV-containing cell line was used as the absorbing material. These absorption studies were performed with undiluted sera. It remains possible that, in those instances where precipitating activity was not removed, repeated absorption with the same materials using sera diluted to the threshold of precipitating activity might have removed activity.

It is highly unlikely that a laboratory contaminating agent could be responsible for the antigenic activity, since the antigen could be prepared from cells harvested in our laboratory over a six month period, as well as from cell lines grown at Chas. Pfizer and Co. High-titered monospecific antisera prepared in animals to various common fungi and Mycoplasma did not contain precipitating antibody to HR1K antigens.

Finally, the correlation of number of precipitin bands with fluorescent antibody titer also suggests the antigens revealed in immunodiffusion are virus specific. Studies on individual cells with the immunofluorescence test\(^{28,29}\) have confirmed that this test is detecting viral antigen. Indeed, the antigen-antibody reaction resulting in fluorescence of infected cells may be a summation of several different antibodies reacting with several different virus associated antigens, which are separated in immunodiffusion into distinct precipitin reac-
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The results of the electron microscopic and trypsinization studies and the finding of identity of soluble cell-derived antigen and purified supernatant virus antigen were of interest. These results suggest that the precipitin reactions using purified virus as antigen (ref. 6, the present study) result from protein components of the virion diffusing into the agar. This could result from degradation of the virus at room temperature in the antigen well, and if so, would thus be analogous to immunodiffusion characteristics described for purified murine leukemia viruses.27

In conclusion, it appears that some previous methods of immunodiffusion with P3J antigens5,21 detect the incidence in peak serum reactors, and this would explain the higher incidence of antibody recorded in some patient groups compared to others. For example, Old et al.22 reported an incidence of precipitating antibody in patients with Burkitt's lymphoma of 59 per cent compared with an incidence of eight per cent in healthy Americans and patients with nonmalignant diseases. The prevalence of precipitating antibody reported in this study (e.g., 92 per cent of Burkitt lymphoma patients, 46 per cent of all American controls) appears to approximate that detected by other immunological methods,2,3,5 and is not disease-specific. We suggest that these differences are due to increased sensitivity with the methods described here, but further studies are needed. The high antibody activity in some disease states (e.g., Burkitt's lymphoma) detected in other methods2,3,5,8 by titration may be reflected in this test in increased number of precipitin bands, probably by precipitation of several weaker antigens. With any of these serological tests, however, interpretation must be cautious regarding comparison of a disease group of varying clinical and therapeutic status and a group of normal individuals not matched for age, region, socioeconomic status, etc. The presence of antibody in early childhood as detected by methods reported here is likewise in agreement with data obtained by other serological methods.

Whether the serological differences described reflect an etiological role for this virus or a predilection for multiplication in abnormal cells associated with lymphoproliferative diseases remains to be determined. Continued interest in this virus is anticipated in view of the association of viruses of the same family with animal neoplasms, including avian neurolymphomatosis,20 lymphosarcomas of toads,21 rabbit lymphoma,22 simian reticulum cell sarcoma,23 and renal adenocarcinomas of frogs,24 and with cervical cancer in man.25

SUMMARY

The development of a sensitive immunodiffusion test for precipitating antibodies to antigens derived from herpes-type virus (HTV) infected Burkitt lymphoma cells in tissue culture is described. Seroepidemiologic observations with 517 sera were made. Precipitating antibody was found in a majority of the normal adult population, in contrast to some previous reports. The number of precipitin bands obtained was directly related to the titer of antibody reacting in indirect immunofluorescence. Antibody activity appears markedly increased in the sera of patients with Burkitt's lymphoma, as shown
by percent positive and number of precipitin bands. Studies of the antigens suggest that the precipitins are virus-specific, and may be virion protein antigens.

ACKNOWLEDGMENT

The authors would like to express their appreciation to Dr. Mary A. Fink, Dr. Robert Manaker, and Dr. Albert Dalton, National Cancer Institute, for review of the manuscript; to Mrs. S. Einbinder for assistance in preparation of the manuscript; and to the physicians, investigators, and blood bank personnel, too numerous to mention individually, who generously provided the serum specimens used in this study.

REFERENCES

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Prevalence of Precipitating Antibody to Antigens Derived from Burkitt Lymphoma Cultures Infected with Herpes-Type Virus (EB Virus)

DAVID A. STEVENS, THOMAS W. PRY and ELIZABETH A. BLACKHAM