AIDS* has become a major epidemic in the United States (1). The overall mortality from the associated Kaposi's sarcoma and opportunistic infection is 40%. Approximately 3200 cases have been described in the United States in the last 3 years. The immunodeficiency has been well described in these patients. Recently, it has become evident that a much larger number of individuals have a disease entity which may precede the development of AIDS (21). This is a syndrome characterized by fever, night sweats, lymphadenopathy, weight loss, diarrhea, and fatigue with similar immunological findings to those seen in AIDS. This has been referred to as the ARC. In addition, it appears that patients in at least one of the high-risk groups for the development of ARC and AIDS may also have similar immunological abnormalities (17). Some of the abnormalities include impaired delayed hypersensitivity, low relative and absolute levels of helper cells, inverted helper:suppressor ratio, impaired lymphocyte blastogenesis, impaired NK cell activity, elevated serum thymosin-α1 (9), serum β2-microglobulin (26), serum lipoprotein (17), hypergammaglobulinemia, and circulating immune complexes (18). One of the prime immunological defects appears to be the low peripheral blood T-helper cells with an inverted ratio of T-helper to T-suppressor cells.

The etiology of the disease is unknown, but a transmissible viral agent superimposed on a preexisting immune deficiency has been hypothesized. A leading candidate at present is human T-cell leukemia virus (2, 4, 5). The fundamental mechanism of the immunodeficiency is also not well understood but presumably relates to a direct immunosuppressive effect of the virus. The consequences, of course, are the development of opportunistic infection and unusual cancers.

Several workers have attempted to investigate the mechanism and have demonstrated recently impaired IL-2 production and response (25), impaired helper cell activity with impaired immunoglobulin synthesis (11), and augmented suppressor cell activity (7). In addition, impaired production of interferon upon in vitro stimulation with herpes simplex viruses has been noted in AIDS patients (14). Because of the high incidence of opportunistic viral infections in these patients, associated with T-cell immunodeficiency and the above-noted findings, we elected to further study the mechanism by characterizing the in vitro response to 2 viruses which have been associated with AIDS. Peripheral blood leukocytes from patients were exposed in vitro to CMV and HSV, and the lymphocyte-proliferative responses, the generation of NK cells, and the induction of interferon were studied. Also, the effects of IL-2 added in vitro on these parameters were also studied.

MATERIALS AND METHODS

Twenty patients were studied. They were chosen at random from a group of 300 sexually active homosexual men between the ages of 20...
and 50 being followed in the Cancer Prevention Clinic at M.D. Anderson Hospital and Tumor Institute. None was receiving treatment at the time of study. Forty-four had AIDS; 4 had ARC; and 2 were symptom free, had never had AIDS-related symptoms or significant prior medical histories. These symptom-free patients were included, since we have demonstrated that these high-risk patients have the same type of abnormalities as ARC and AIDS patients. Eighteen heterosexual normal controls, aged 20 to 50, were also studied. None of the patients was on therapy nor had any received prior therapy for AIDS. The individual controls were studied concurrently with one or two of the patients studied.

The viral materials were prepared as follows. HSV-1, strain 539, and HSV-2, strain 316-D, were replicated in rabbit skin cells to a titer of approximately 2 x 10^8 plaque-forming units/ml. Cellular debris was removed by centrifugation at 2500 x g for 30 min. Supernatant fluid was heated at 56°C for 60 min to inactivate virus. The virus pool was aliquoted in 1-ml volumes and stored at -70° until used. Control antigen consisted of supernatant fluid from rabbit skin cells treated identically to the virus preparation.

16-CMV and 17-CMV control antigen were prepared as follows. A stock laboratory strain of 17-CMV (AD1169) was grown to confluence in human foreskin fibroblast-containing flasks. Cellular debris was removed by centrifugation at 2500 x g for 30 min. Supernatant fluid was heated at 56°C for 30 min to inactivate the virus. Aliquots of the virus pool were stored at -75° until used. Control antigen was prepared from uninfected human foreskin fibroblast monolayers that received the same treatment as virus-infected cells.

Lymphocyte cultures were set up as follows (8). Peripheral blood was obtained by venipuncture and defibrinated by swirling with glass beads. RBC were removed, and peripheral blood mononuclear cells were prepared by Ficoll-Hypaque density solution centrifugation. Leukocyte suspensions were washed 3 times in HBSS and resuspended in RPMI 1640 medium with 10% fetal bovine serum. Cells were cultured in round-bottomed Microtest II plates (Falcon). Each culture contained 1.5 x 10^6 peripheral blood mononuclear cells and 0.2 ml of complete medium. Lymphocytes were cultured either unstimulated or stimulated with PHA, CON-A, PWM, CMV control antigen, CMV, HSV control antigen, or HSV-1, strain 539, and HSV-2, strain 316-D. The antigen doses used had been demonstrated previously in preliminary dose-response studies, to be optimal for the induction of lymphocyte proliferation. The antigen dose was approximately 7 x 10^6 viral plaque-forming units/ml of lymphocyte culture. Selected cultures were also treated with partially purified IL-2 obtained from Cellular Products, Inc. (Buffalo, NY) (10% added by volume) or from Immunomodulator Laboratories, Inc. (Houston, TX) (5 growth-supporting units/ml added). These concentrations had been shown previously to optimally maintain proliferation of IL-2-dependent T-cell lines. Cultures were incubated for 4, 24, or 48 hr or 5 days at 37°C in 5% CO2 in air and were assessed at appropriate times for lymphocyte blastogenic responses, NK cell activity, and interferon titer.

Blastogenic responses were measured after 5 days of culture by adding 1 μCi of [3H]thymidine 8 hr prior to harvest with multiple-sample harvesting. Incorporated radioactivity was counted in a liquid scintillation counter. Results were recorded as net cpm per well (12). NK cell activity was measured by a standard chromium release assay as follows (8). The K562 cell line was used as a target cell. Effector:target ratio was 25:1 with 10^6 effector cells per well. This ratio was selected because preliminary experiments showed it to be optimal. Other ratios were not studied. Effector cells were also obtained from 4-, 24-, and 48-hr control, control antigen, and virus-stimulated cultures. Target cells were labeled after washing 3 times in HBSS by incubation for 30 min at 4°C with 100 μCi of 51Cr and washing twice. Cocultures of target and effector cells were incubated for 4 hr at 37°C in an atmosphere of 5% CO2 in air. Supernatants were obtained from these cultures, and the percentage of target cell lysis was calculated.

Serum interferon titers were measured by a bioassay cytotoxic effect, as described previously, using human WISH cells as targets of encephalomyocarditis virus.

Interferon titers were expressed as reciprocals of the dilutions producing a 50% reduction of virus cytopathic effect. All samples were corrected to a human interferon-α standard (G023-901-527 reagent from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) (19).

At the approximate time of their evaluations, all patients had complete immunological evaluations as described previously (17). These included delayed hypersensitivity skin tests, enumeration of leukocyte cell surface markers (including T11, T3, T4, T8, and T10) using Orthomune reagents, and measurement of serum lysozyme and serum thymosin-α1. In addition, a complete laboratory evaluation including complete blood count, blood chemistries, H-CMV titer, and Epstein-Barr virus titers was performed. These data were utilized to correlate the results obtained in the study with the clinical and immunological status of the patients.

Significance of difference between study groups was tested by the Kursral-Wallace one-way analysis of variance. Differences between treated and untreated cultures were tested by the Wilcoxon signed rank test, while correlations of various parameters were calculated by the Spearman rank correlation test.

RESULTS

Table 1 shows the lymphocyte blastogenic responses to the control antigen preparations, the viruses, and the mitogens. The control antigen preparations induced little, if any, blastogenic response. The majority of patients' and control subjects' cells showed some degree of blastogenic response to the virus preparations. The responses of the control subjects' cells to virus were significantly higher than those of the patients only for HSV-1 but not for HSV-2 or for 16-CMV. In contrast, the PHA, CON-A, and PWM responses of the control subjects' cells were significantly higher than those of the patients'. The virus responses were modest, being in the 1000 to 6000 average cpm range, compared to the 30,000 to 60,000 average cpm range for PHA.

Table 2 shows the interferon titers at 24 and 120 hr. Both the patients' and controls' cells showed only a very weak interferon response to CMV. The normal subjects' cells showed a vigorous interferon response to both strains of HSV. It was significantly less by the patients' cells. This was true in both the 24- and 120-hr cultures. Thus, there was a major deficiency in the interferon induction in the patients' cells.

Table 3 shows the relevant NK cell data. Peripheral blood leukocytes were incubated for 4 and 24 hr without any additives and in the presence of viral control antigen or the viral preparations. In general, the patients' unstimulated cells showed significa-

Table 1
Mean in vitro blastogenic responses (cpm x 10^3/culture) to virus-associated antigenic preparations and mitogens in AIDS patients and normal subjects

<table>
<thead>
<tr>
<th>Culture stimulant</th>
<th>Patients</th>
<th>Normals</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV control antigen</td>
<td>0.32</td>
<td>0.43</td>
<td>NS*</td>
</tr>
<tr>
<td>HSV-1</td>
<td>1.75</td>
<td>6.11</td>
<td>0.05</td>
</tr>
<tr>
<td>HSV-2</td>
<td>2.07</td>
<td>6.36</td>
<td>NS</td>
</tr>
<tr>
<td>&quot;-CMV control antigen</td>
<td>0.27</td>
<td>0.48</td>
<td>NS</td>
</tr>
<tr>
<td>&quot;-CMV</td>
<td>3.13</td>
<td>3.38</td>
<td>NS</td>
</tr>
<tr>
<td>Unstimulated control</td>
<td>0.30</td>
<td>0.45</td>
<td>NS</td>
</tr>
<tr>
<td>PHA</td>
<td>38.26</td>
<td>62.05</td>
<td>0.001</td>
</tr>
<tr>
<td>CON-A</td>
<td>13.32</td>
<td>32.35</td>
<td>0.001</td>
</tr>
<tr>
<td>PWM</td>
<td>5.08</td>
<td>22.10</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*NS, not significant.

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the responses to the virus of the normals' lymphocytes, presum-
and virus-stimulated cultures of the patients. In contrast, it
(Table 4). IL-2 added at the initiation of the cultures markedly
the HSV- and H-CMV-treated cultures, suggesting that NK acti-
cultures treated with IL-2 were only slightly lower than those in
one normal subject was similarly boosted. NK cell activity was
3 patients by at least 2 serial 2-fold dilutions. In contrast, only
on the average. However, in the patients, IL-2 boosted the HSV-
not boost the interferon responses of the patients or the normals
ably because these responses were already vigorous. IL-2 did
boost the control antigen-stimulated cultures but did not boost
activation between these titers and the degree of response of the
ranged from 1:64 to 1:4096 with a median of 1:1024, and all but
4 of the patients had an EBV titer which ranged from 1:160 to
1:2560 with a median of 1:640. There was no significant corre-
lationship between these titers and the degree of response of the
patients to either virus in terms of blastogenesis, NK cell activa-
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The addition of IL-2 to virus-stimulated cultures was studied
(Table 4). IL-2 added at the initiation of the cultures markedly
boosted the blastogenic responses of the nonantigen-stimulated
and virus-stimulated cultures of the patients. In contrast, it
boosted the control antigen-stimulated cultures but did not boost
the responses to the virus of the normals' lymphocytes, presum-
ably because these responses were already vigorous. IL-2 did
not boost the interferon responses of the patients or the normals
on the average. However, in the patients, IL-2 boosted the HSV-
1 interferon responses in 4 patients and the HSV-2 responses in
3 patients by at least 2 serial 2-fold dilutions. In contrast, only
one normal subject was similarly boosted. NK cell activity was
markedly boosted in patients and normals. However, the pa-
patients' boosted NK levels were still below those of the normals.
Furthermore, the levels of NK activity in the control antigen
cultures treated with IL-2 were only slightly lower than those in
the HSV- and H-CMV-treated cultures, suggesting that NK acti-
vation by virus or IL-2 was near maximal, and there was minimal,
although significant, additive effect.

Table 2
Mean interferon levels (IU/ml) in culture supernatants of leukocyte cultures
stimulated in vitro with virus-associated antigenic preparations in AIDS patients
and normal subjects

<table>
<thead>
<tr>
<th>Culture stimulant</th>
<th>24-hr cultures</th>
<th>120-hr cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Normals</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSV control antigen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSV-1</td>
<td>138.8</td>
<td>776.5 &lt;0.001</td>
</tr>
<tr>
<td>HSV-2</td>
<td>118.0</td>
<td>564.7 &lt;0.001</td>
</tr>
<tr>
<td>H-CMV control antigen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H-CMV</td>
<td>5.9</td>
<td>18.6 0.036</td>
</tr>
</tbody>
</table>
* NS, not significant.

Table 4 shows the correlations of the various immunological
parameters with the responses to HSV-2. Correlations with the
responses to HSV-1 were similar and are not shown. Only
significant correlations are shown. Significant correlations were
observed between the NK cell activity, interferon production, and
the blastogenic responses. The response parameters for each
virus also correlated significantly with the level of lymphocyte or
T-cells, the level of helper cells, the helper:suppressor ratio, and
the mitogen responses. Boosting of NK cell activity and interferon
production was also correlated in normals (data not shown).

For each of the major parameters of response to virus (blas-
togenic response, NK cell response, and interferon production),
the 6 symptom-free and ARC patients were compared to the 14
AIDS patients. There were no differences between these 2
groups (data not shown), suggesting that the defects in re-
sponses to the virus were as severe in early patients as in those
with advanced disease.

All patients but one had a positive antibody titer to CMV
(measured within 4 weeks of the studies described herein), which
ranged from 1:64 to 1:4096 with a median of 1:1024, and all but
4 of the patients had an EBV titer which ranged from 1:160 to
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lolation of these titers and the degree of response of the
patients to either virus in terms of blastogenesis, NK cell activa-
tion, or interferon induction.

DISCUSSION

A number of groups have focused studies of AIDS on the
function of various leukocyte subsets. Evidence has already been
developed which suggests that various cytokines, such as IL-2,
may be produced in abnormally low levels by the lymphocytes
patients with AIDS and that their impaired immunological
function in vitro may even be corrected by exogenous adminis-
tration of IL-2 (25). While these AIDS patients often manifest an
abnormal acid-labile circulating interferon-α (19), the possibility
exists that they are not capable of producing interferon normally,
since they have a high susceptibility to viral infection. This has
been confirmed in one recent report (14). A generalized failure
of cytokine production might be responsible for the impaired
lymphocyte proliferation, the low levels of NK cells, as well as the
impaired in vivo delayed hypersensitivity.

Table 3
Stimulation of increased NK cell activity by virus-associated antigens in AIDS patients and normal subjects

<table>
<thead>
<tr>
<th>Culture additives</th>
<th>Patients</th>
<th>Normals</th>
<th>p</th>
</tr>
</thead>
</table>
|                   | % of cyto-
|                   | Significance | % of cyto-
|                   | Significance | p  |
|                   |          |         |    |
| Control           | 6.1      | 11.7    | 0.003 |
| HSV control antigen | 5.8      | 14.6    | 0.007 |
| HSV-1             | 5.9      | 17.9    | 0.005 |
| HSV-2             | 6.9      | 18.8    | 0.005 |
| H-CMV control antigen | 4.1      | 11.0    | 0.018 |
| H-CMV             | 3.7      | 12.4    | 0.002 |
* NS, not significant.

Table 5 shows the correlations of the various immunological
parameters with the responses to HSV-2. Correlations with the
responses to HSV-1 were similar and are not shown. Only
significant correlations are shown. Significant correlations were
observed between the NK cell activity, interferon production, and
the blastogenic responses. The response parameters for each
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Table 3
Stimulation of increased NK cell activity by virus-associated antigens in AIDS patients and normal subjects

<table>
<thead>
<tr>
<th>Culture additives</th>
<th>4-hr cultures</th>
<th>24-hr cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of cytotoxicity</td>
<td>Significance</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.1</td>
<td>11.7</td>
</tr>
<tr>
<td>HSV control antigen</td>
<td>5.8</td>
<td>14.6</td>
</tr>
<tr>
<td>HSV-1</td>
<td>5.9</td>
<td>17.9</td>
</tr>
<tr>
<td>HSV-2</td>
<td>6.9</td>
<td>18.8</td>
</tr>
<tr>
<td>H-CMV control antigen</td>
<td>4.1</td>
<td>11.0</td>
</tr>
<tr>
<td>H-CMV</td>
<td>3.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>
* NS, not significant.

Data shown are mean values.
The results reported in this paper indicate that there is indeed impaired, but measurable, lymphocyte proliferation in these patients to the viruses used. Exposure of their lymphocytes to viruses associated with AIDS (such as HSV and CMV) does not, however, induce normal lymphocyte production (14) and also does not induce normal IL-2 production (25). Failure of production of these 2 cytokines would explain the lack of NK cell boosting in these patients. IL-2 production was induced (3). It is known that interferon-$
abla$
 production itself, or a failure of the cellular defense mechanism directed at virus-infected or -transformed cells, might facilitate the proliferation of a variety of viruses possibly related to AIDS or AIDS-related Kaposi’s sarcoma or central nervous system lymphoma (10, 20, 23).

**REFERENCES**


Impaired *in Vitro* Interferon, Blastogenic, and Natural Killer Cell Responses to Viral Stimulation in Acquired Immune Deficiency Syndrome

Evan M. Hersh, Jordan U. Gutterman, Steven Spector, et al.


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