NATURAL INHIBITORS OF T-CELL ACTIVATION IN HODGKIN’S DISEASE

By Matthias Roux, Burkhart Schraven, Albert Roux, Heinold Gamm, Roland Mertelsmann, and Stefan Meuer

Secondary immunodeficiency is frequently observed in Hodgkin’s disease (HD) and is due in part to impaired T-cell function. Using monoclonal antibodies that bind to triggering molecules of human T lymphocytes (CD3/Ti antigen receptor; CD2 E-rosette receptor) and exert functional effects on T-cell activation, we have investigated in vitro immune responses of circulating lymphocytes from patients with HD in progression (n = 9) and in remission (n = 14). In patients with progressive HD, a severe dysfunction of the alternative CD2-mediated T-cell activation pathway was detected (49.3 ± 14.2 vs 9.4 ± 5.1 cpm × 10^{-3}, in controls, P < .01; n = 9) that parallels the reduced capacity of T lymphocytes to form rosettes with sheep red blood cells. Diminished alternative pathway activation in HD is not only due to a defect at the cellular level but also due to soluble mediators in the patients’ plasma. Plasma from patients in progression markedly reduces CD2 mediated activation (P < .01). These activities interfere, at least in part, with CD2/CD58 interactions and, therefore, reduce T-lymphocyte triggering through this amplifier mechanism.

MATERIALS AND METHODS

Patients. The 23 patients investigated suffered either from HD in progression (n = 9) or in remission (n = 14). Staging was performed according to the “Hodgkin’s Disease Staging Classification Committee” (Table 1). None of the patients received any therapy at the time of investigation. The control subjects (n = 9) were healthy members of the laboratory staff (age, 25 to 32 years, four women, five men).

Preparation of lymphocytes and plasma. Heparinized blood samples were spun for 20 minutes at 460g to separate plasma. Plasma was then heat-inactivated for 30 minutes at 56°C and then spun at 3,300g for 20 minutes to remove aggregates. The supernatant was stored at 5°C. The cell pellet was resuspended in RPMI 1640 (GIBCO, Paisely, Scotland), 2% L-glutamine (200 mmol/L; GIBCO), 1% penillicin-streptomycin (10,000 IU/mL penicillin, 10,000 μg/mL streptomycin; GIBCO), and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque (Pharma- cia, Upsalsal, Sweden) density centrifugation. Subsequently, PBMC were dissolved in RPMI 1640, 2% L-glutamine, and 1% penicillin-streptomycin supplemented with 15% of the indicated plasma and kept at 37°C, 7% CO₂, 100% humidity for 2 hours before the experiments.

Purified T cells were prepared as follows: PBMC from healthy donors were mixed with sheep erythrocytes (20 μL of a 5% vol/vol suspension/10^7 PBMC), spun at 73g for 5 minutes, and then incubated for 1 hour at room temperature. Subsequently, cells were gently resuspended, the suspension underlayered with Ficoll-Hypaque (Pharmacia), and spun for 20 minutes at 200g and for 15 minutes at 460g. Sheep erythrocytes bound to the pelleted rosetting T cells were then lysed with ACK solution (155 mmol/L NaCl, 10 mmol/L KHCO₃, 0.1 mmol/L K-EDTA). Separated T cells were twice washed with RPMI 1640, 2% L-glutamine, 1% penicillin-streptomycin, and 5% human serum and incubated for 45 minutes.

From Abteilung Angewandte Immunologie, Deutsches Krebsforschungszentrum, Heidelberg; III. Med. Klinik, Johannes Gutenberg-Universität Mainz; and Med. Klinik und Poliklinik, Albert Ludwigs-Universität Freiburg, Germany.


Address reprint requests to Matthias Roux, MD, Deutsches Krebsforschungszentrum, Institut 08, Abteilung Angewandte Immunologie, Im Neuenheimer Feld 280, 6900 Heidelberg, Germany.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.


2365
at 37°C with 100 μL MoAb αHLA II, 100 μL MoAb MO1, and rabbit complement to lyse preactivated T cells and residual monocytes. Cells were then washed twice again before use. This procedure to prepare purified T cells does not influence CD2- or CD3-mediated activation, provided that the SRBC are properly lysed.

Rosetting tests. Thirty microliters of sheep erythrocytes (5% [vol/vol] in RPMI 1640) were added to 10^6 PBMC that had been preincubated for 2 hours in RPMI, 2% L-glutamine, 1% penicillin-streptomycin, and 15% of the indicated plasma at 37°C. Cells were spun at 600 rpm for 5 minutes and allowed to form rosettes at room temperature for 1 hour. Afterwards, the pellet was gently resuspended. Rosetting and nonrossetting cells were separated by Ficoll-Hypaque density centrifugation and counted.

MoAbs and mitogens. MoAbs were used in ascerts form in predetermined concentrations: CD4 (AICD4.1), CD8 (AICD8.2), α T11.1 (AICD2.1.1A), α T11.α (AICD2.2.1B), CD3 MoAb (RW28C8), α T11.1 (10LD4.1C), and α T11.α (IMONO2A6) were kindly provided by Drs Schllossmann and Reinherz (Dana-Faber Cancer Institute, Boston, MA). αHLA II (No. 6) and αCD58^7 were previously established on PBMC of healthy donors.

Purified CD3 MoAb (OKT3; American Type Culture Collection, Rockville, MD) purified by high-performance liquid chromatography from mouse ascites was covalently coupled to cyanogen bromide (CNBr)-activated Sepharose beads (Pharmacia) at a concentration of 5 mg/mL swelled beads according to the instruction of the supplier. The beads were stored in phosphate-buffered saline (PBS)/0.1% NaCl (vol/vol) and extensively washed before use in the proliferation studies.

Phytohemagglutinin (PHA) was purchased from Wellcome (Burgwedel, Germany) and used at a final concentration of 0.1 μg/mL.

Natural interleukin-2 (nIL-2) is a highly purified preparation of human IL-2 (Biogen, Dreieich, Germany).

Recombinant human IL-4 purified from Escherichia coli was a generous gift of Dr J. de Vries (Laboratoire Unicet, Dardilly, France).

The concentrations at which stimuli (PHA, CD3 MoAb-Sepharose beads, αT11.1 + αT11.α, and αT11.α) were used were previously established on PBMC of healthy donors.

Immunofluorescence. Single-color fluorescence flow cytometric analyses were performed on an EPICS 752 cell sorter (Coulter Electronics, Hialeah, FL). Cells (1 × 10^6) were stained for 30 minutes at 4°C with saturating concentrations of MoAb and washed twice with PBS (Seromed, Berlin, Germany), followed by incubation with 50 μL of a 1:60 dilution of goat antismouse fluorescein isothiocyanate-conjugated antibody (Coulter Electronics). Cells were fixed in 0.5 mL PBS/1% (vol/vol) parafomaldehyde. Ten thousand cells were analyzed per sample.

Proliferative assays. PBMC or purified T cells (1 × 10^6), respectively, were cultured in round-bottomed microtiter plates (Costar, Cambridge, MA) in 200 μL RPMI 1640 supplemented with 2% L-glutamine, 1% penicillin-streptomycin, and 15% of plasma as indicated. After 72 hours, individual wells were pulsed with 37 kBq ^3H-thymidine (185 μCi/mL Amersham, Braunschweig, Germany) for 18 hours and harvested using an Inotec cell harvester (Wohlen, Switzerland). ^3H-thymidine incorporation was measured using a liquid scintillation spectrometer (Beckmann, Munich, Germany). Results are expressed as means of triplicates ± SEM. Values of the proliferation assays and the rosetting tests were transformed to logs and compared by t-test analysis.

RESULTS

Rosette-forming assay. PBMC from control subjects form rosettes with sheep erythrocytes at 62% ± 11% when incubated in autologous plasma before the test. For reasons of better comparability, this control value was set at 100% and the respective values of the patients expressed as percentage of the control. The capacity of PB lymphocytes from HD patients in remission to bind to sheep erythrocytes is 97% ± 9.2% as compared with healthy controls (P < .05). In contrast, rosette formation of PBMC from patients with active disease was reduced to 76% ± 6.2% when compared with the control cells (P < .01), confirming data previously described by other investigators.16-20

Immunofluorescence. To investigate whether the reduced capacity of PBMC from HD patients was due to reduced expression of cell surface molecules known to be involved in T-cell activation and SRBC-rosette formation we performed immunofluorescence studies. To this end, 5 × 10^6 cells/sample were labeled with MoAbs directed against the indicated surface molecules (Table 2) and 10^5 cells/sample were analyzed on a flow cytometer.

As shown in Table 2, both groups of patients had slightly reduced numbers of circulating T cells as shown by the diminished count of CD3^+ PBMC. The CD2 epitopes critical for CD2/CD58 interaction (ie, T11.α and T11.α) are expressed. Although not confirmed by dual-fluorescence labeling, this analysis suggests that CD3^+ T cells also bear CD2. In addition, the density of CD2 molecules and/or epitopes on the T-cell surface was not diminished in patients, as calculated from mean channel fluorescence values (data not shown).

Proliferative assays. To investigate the functional capacity of T cells from HD patients to respond to triggering via the TCR, a Sepharose-linked CD3 MoAb (seph-CD3) was used.
MoAb binding to T-cell surface molecules as determined by an EPICS 752 cell sorter (percent positives ± SEM). PBMC of the individual subjects were prepared, incubated with the indicated MoAb, and stained with a goat antimouse fluorescein isothiocyanate-conjugated second antibody as described in Materials and Methods. The negative controls were always less than 3%.

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients in Remission</th>
<th>Patients in Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>68 ± 5.3</td>
<td>46 ± 4.3</td>
<td>52 ± 7.8</td>
</tr>
<tr>
<td>CD4</td>
<td>47 ± 3.6</td>
<td>32 ± 4.8</td>
<td>32 ± 5.4</td>
</tr>
<tr>
<td>CD8</td>
<td>25 ± 2.1</td>
<td>24 ± 2.3</td>
<td>23 ± 4.9</td>
</tr>
<tr>
<td>Ratio CD4/CD8</td>
<td>2.0 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>CD2.1A</td>
<td>82 ± 4.8</td>
<td>59 ± 5.2</td>
<td>57 ± 10.9</td>
</tr>
<tr>
<td>CD2.1B</td>
<td>78 ± 3.3</td>
<td>57 ± 4.2</td>
<td>62 ± 8.4</td>
</tr>
<tr>
<td>CD2.2</td>
<td>76 ± 3.7</td>
<td>58 ± 5.0</td>
<td>61 ± 8.6</td>
</tr>
<tr>
<td>CD2.3</td>
<td>14 ± 2.2</td>
<td>28 ± 3.3</td>
<td>16 ± 5.3</td>
</tr>
<tr>
<td>CD58</td>
<td>11 ± 4.6</td>
<td>18 ± 2.1</td>
<td>7 ± 5.4</td>
</tr>
<tr>
<td>HLA II</td>
<td>8 ± 1.5</td>
<td>17 ± 1.9</td>
<td>15 ± 3.6</td>
</tr>
</tbody>
</table>

To determine whether the deficiency in T-lymphocyte activation was due to a defect at the cellular level or due to factors present in the patients' plasma, we performed proliferation assays with T lymphocytes from a healthy subject and plasma either from a second healthy donor or from patients with progressive HD. As shown in Fig 2, when compared with plasma from a healthy donor, plasma from HD patients reduced proliferation of PBMC from healthy individuals to a similar degree in the stimulation systems used as previously observed with PBMC of HD patients. Note that T-cell triggering via CD3 resulted in a lower 3H-thymidine incorporation than stimulation via CD2 and should therefore be more susceptible to blocking influences. Nevertheless, triggering by MoAb T111 + T11 was reduced by 80% (P < .01), whereas CD3 triggering was only slightly diminished (P > .05). Again, this predominant inhibition of CD2-mediated T-cell proliferation could be partially circumvented in the presence of αT11, MoAb (59% of control; P < .05) (Fig 2).

Activation of T cells by seph-CD3 MoAbs requires the presence of monocytes to induce IL-2 production. In contrast, IL-2 responsiveness (ie, IL-2 receptor expression) can be induced after seph-CD3 triggering even in the absence of monocytes. Therefore, it is likely that, in addition to signals mediated via the CD3-Ti antigen receptor, the CD3 system may also contain activation stimuli delivered through CD2/CD58 expressed on the proportion of activated T cells and monocytes contained in PBMC, respectively. It follows that the inhibitory effect on T-cell activation by seph-CD3, observed above by plasma from HD patients, could have been only due to interference with CD2/CD58 binding and not with the signals transmitted through the T-cell receptor itself. To investigate this point, we established a modified CD3-triggered proliferation system in which signals delivered via the CD2 molecule were excluded. To this end, resting T cells were vigorously purified. As a control of proper purification, T-cell proliferation must not be induced by either CD3, PHA, or IL-2 alone, as shown by others. T cells were activated by seph-CD3 MoAb plus IL-2 in the presence of a MoAb directed at the T111B epitope (MoAb AICD2.2.1B) (Fig 3). This antibody selectively inhibits binding of CD58 to CD2 without providing a functional signal. In line with the above considerations, blocking of CD2/CD58 binding by MoAb AICD2.2.1B abrogated the activation by seph-CD3 plus IL-2 to a substantial degree. Nevertheless, plasma from HD patients still reduces the residual proliferation by greater than 50%. Note that this inhibition could be circumvented neither by the addition of IL-2 or IL-4 (Fig 3).

**DISCUSSION**

Besides triggering through the CD3-Ti antigen receptor complex, T lymphocytes can be activated by MoAbs directed at the SRBC receptor now termed CD2. Physiologic ligand of CD2, CD58, has been identified. The interaction between CD2 and CD58 has been shown to be essential for optimal antigen receptor-mediated signaling. Moreover, it has been shown that binding of CD2 to CD58 delivers a triggering signal to T cells, which leads to polyclonal T-lymphocyte activation in combination with antibodies directed at distinct epitopes of CD2. Moreover, blockade of CD2/CD58 binding inhibits T-cell activation in a number of immunologic processes, eg, mixed
lymphocyte reaction and antigen-driven proliferation. This finding suggests that CD2/CD58 interactions are involved in physiologically occurring immune responses.

Formation of rosettes between T cells and SRBC mimics CD2/CD58 binding because this reaction is mediated mainly through CD2 expressed on T lymphocytes and T11 Ts, the sheep homologue of CD58 on SRBC. In HD, a reduced E-rosette-forming capacity of T cells has been well documented. Given the in vitro functional importance of CD2/CD58 interaction for the generation of specific immune responses, a defect of T-cell activation might exist at this level in vivo as well. This defect could be responsible in part for the severe immunodeficiency existing in HD.

In this report we show that the diminished E-rosette formation in patients with active HD results in a nearly abolished capacity of T cells to respond to CD2 triggering. This effect is obviously not due to a primary defect at the cellular level but rather is related to inhibitory activities in the plasma from HD patients, because the expression of CD2 on T cells of HD patients is hardly altered. This view is further supported by experiments (Figs 2 and 3) in which an MoAb directed at one CD58 binding site on CD2 (T11.1a) partially restores the capacity of lymphocytes from HD patients to proliferate to CD2-dependent stimuli. It seems possible that due to its high binding affinity this antibody (aT11.1a) outcompetes circulating blocking factors in HD plasma that might have bound to the CD2 molecule of patient T cells. Whether these "factors" in the patients'
plasma are tumor cell products or, alternatively, physiologic activities induced in the course of the disease process is not known at present. It is not unlikely that a soluble form of one of the ligands involved in CD2/CD58 interaction exists in the sera of patients with HD. Besides a transmembrane form of CD58, a phosphatidylinositol (PI)-anchored form of this molecule could be identified. This finding makes it likely to be released from the cell surface by specific phospholipases, as could be shown in vitro. We are now establishing an enzyme-linked immunosorbent assay (ELISA) system to determine whether soluble forms of CD58 and CD2 exist.

Besides CD2-mediated activation, T-cell triggering via the antigen receptor was also partially inhibited. This result was shown in a system in which the proportion of CD3-driven activation that likely depends on the interaction between CD2 and CD58 was essentially diminished. This finding suggests that, besides factors that interfere with CD2/CD58 binding, inhibitors of T-cell proliferation are present in the HD patients’ plasma that affect T-cell acti-
vation through CD3 at a distinct level. The finding that these inhibitory activities cannot be circumvented by the lymphokines IL-2 and IL-4 suggests a defect in the signalling process required for antigen receptor-mediated stimulation.

In conclusion, this study indicates that reduced E-rosette formation of T cells in HD represents a marker for diminished T-lymphocyte activation via the CD2-mediated "alternative pathway." Thus, this study presents for the first time evidence that defects in amplifier mechanisms, such as CD2 triggering of T-cell activation, may contribute to the development of secondary immunodeficiencies in vivo. It is tempting to speculate that this finding could be of potential clinical significance in other diseases in which reduced E-rosette formation has been described as well.

REFERENCES


29. Peterson A, Seed B: Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). Nature 329:842, 1987


Natural inhibitors of T-cell activation in Hodgkin's disease

M Roux, B Schraven, A Roux, H Gamm, R Mertelsmann and S Meuer

Updated information and services can be found at:
http://www.bloodjournal.org/content/78/9/2365.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml