CONCISE REPORT

High Numbers of CD4+ T Cells Showing Abnormal Recognition of DR Antigens in Lymphoid Organs Involved by Hodgkin’s Disease

By E. Maggi, P. Parronchi, D. Macchia, G. Bellesi, and S. Romagnani

Purified T lymphocytes (E rosetting cells) isolated from the involved lymphoid organs (lymph nodes and spleen) of five patients with Hodgkin’s disease (HD) were cloned under culture conditions (phytohemagglutinin plus interleukin-2) that allow clonal expansion of most T lymphocytes. A total number of 104 CD4+ T cell clones so obtained were tested for their ability to proliferate in response to autologous mitomycin-treated non-T cells. About half of these clones but none of 234 CD4+ T cell clones derived from normal lymphoid tissues or peripheral blood displayed a proliferative response to autologous stimulators. When clones proliferating in autologous mixed lymphocyte reaction (AMLR) were assessed for their ability to respond in allogeneic MLR (allo-MLR), most of them were found to exhibit consistent proliferation in response to more than one haplotype. Both the AMLR and the allo-MLR by HD clones were inhibited by adding monoclonal antibodies (MoAbs) reactive with monomorphic determinants of major histocompatibility complex (MHC) class II (DR) antigens to the cultures, whereas MoAbs reactive with MHC class I antigens were without effect. These studies suggest that lymphoid organs involved by HD contain high proportions of CD4 T cells showing abnormal recognition of DR antigens. These unusual cells may play an important role in the pathogenetic mechanisms occurring in HD.

Unlike the non-Hodgkin’s lymphomas where the predominant neoplastic clone is usually evident, the proliferation in lymphoid organs involved by Hodgkin’s disease (HD) is far more complex. The origin and biologic function of the Reed-Sternberg (R-S) cell, the diagnostic cell in HD, is still unknown. Moreover, the R-S cell is present in small numbers among a complex mixture of apparently normal cell types. Lymphocyte surface findings have shown that the majority of cells infiltrating lymphoid organs involved by HD expressed the CD4 antigen,1 which defines the helper/inducer T cell population.2 However, the role of associated lymphocytes in organs involved by HD has remained an enigma despite a considerable number of immunologic and cell culture analyses.

In this study we attempted to determine the actual functional composition of CD4+ T cell populations infiltrating lymphoid organs involved by HD by the application of high cloning efficiency techniques. We detected unexpectedly high percentages of autoreactive CD4+ T cell clones in four lymph nodes (LNs) and one spleen involved by HD. In addition, the majority of these clones were also found to proliferate in response to a panel of different allogeneic haplotypes.

MATERIALS AND METHODS

Isolation of lymphoid cells. Lymphocytes were isolated from the LN or spleen material of six patients with HD by Ficoll/Hypaque gradients as reported.3 The lymphoid tissue was obtained by surgical procedures performed for diagnostic purpose and was found to be histologically involved by the disease (nodular sclerosis in three LNs and mixed cellularity in one LN and the two spleens, according to the classification of Lukes et al.4) The pathological stage was II A in three patients and III A, III A, and III B in the other three patients. As controls, lymphocytes were isolated from the LNs removed from two patients with nonspecific hyperplastic lymphoadenitis, normal spleens obtained from three subjects who underwent posttraumatic splenectomy, tonsil obtained from a child subjected to tonsillectomy for chronic tonsillitis, and peripheral blood (PB) from three normal donors.

T cells were further purified by plastic adherence and by rosette formation with neuraminidase-treated sheep erythrocytes (E) and subsequent centrifugation (at least two) over standard Ficoll/Hypaque density gradients as described.1

T cell cloning procedure. T cells were plated under limiting numbers in round-bottomed microtiter plates containing 10^5 irradiated (5,000 rad) allogeneic spleen feeder cells and 1% phytohemagglutinin (PHA Gibco, Grand Island) plus recombinant interleukin-2 (r IL-2, kindly provided by Biogen, Geneva) as previously described.5 After 14 to 18 days of culture, the number of wells containing colonies were scored, and the percentage of negative cells was calculated. The average number of cells needed to generate a single clone (precursor frequency) was determined by plotting the number of cells plated per well against the logarithm of the percentage of negative wells; according to one-hit Poisson statistics,5 the precursor frequency is equal to the cell density that would yield 37% negative wells. For further study, only colonies having a 90% probability of true clonality by Poisson statistics were expanded. In two cases (LN 110 and LN 115) the same cells were also cultured under limiting-dilution conditions in a setting similar to the one described earlier but without the addition of PHA. In this case IL-2 was added at the initiation of the culture.

Monoclonal antibodies and surface marker analysis. The surface antigen expression of purified T lymphocytes or clones was studied by staining with OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8) monoclonal antibodies (MoAbs; Ortho Pharmaceutical Corp, Raritan, NJ) and a fluorescein isothiocyanate–conjugated rabbit antimouse IgG. Positivity was assessed by fluorescence microscope examination as previously described.3

CR10-214 and CR11-115, MoAbs to distinct monomorphic determinants of HLA class I antigens, and CL413 and PTF29.12,
MoAbs to monomorphic determinants of HLA-DR antigens, were kindly provided by Drs S. Ferrone (Department of Microbiology and Immunology, New York Medical College) and G. Damiani (Istituto di Biochimica, Università di Genova). Fab(1g)2 fragments were obtained by pepsin digestion from the MoAb PTF29.12 and were purified as previously described.

**Proliferation assays.** Cloned T cells (responder cells) were mixed into 96-well plates (Costar, Cambridge, MA) at a density of 2 x 10⁴/well with mitomycin-treated (50 μg/mL/10⁶ cells) autologous or allogeneic stimulator cells (non-T lymphocytes and lymphoblastoid cell lines) in RPMI 1640 containing 10% heat-inactivated human AB serum. Non-T cells were obtained from PB or lymphoid organ suspensions by repeating the E rosette technique twice as described. E rosetting (T) and nonrosetting (non-T) cells were separated by centrifugation on a Ficoll-Hypaque gradient. B lymphoblastoid cell lines were established by Epstein-Barr virus (EBV) transformation of B cells isolated from PB or lymphoid organs as described.

The optimal responder-to-stimulator ratios, predetermined by testing several stimulating cell doses, were 4:1. The mixed lymphocyte cultures (MLC) were incubated for three days at 37°C, and proliferative responses were determined by measuring [³H]-thymidine incorporation as described.

When the stimulation index (SI; ratio between the mean counts per minute obtained in MLC and the sum of the mean counts per minute obtained in cultures containing responder or stimulator cells alone) was equal to or greater than 10, responses were considered positive.

Stimulation inhibition experiments were performed by adding anti-HLA class I or class II MoAbs to the MLC at the indicated times and concentrations.

**RESULTS AND DISCUSSION**

In all, 104 CD3+, CD4+, CD8− T cell clones were established from the lymphoid organs involved by HD under culture conditions (PHA plus IL-2) allowing clonal expansion of most T cells. As shown in Table 1, the cloning efficiency ranged from 25% to 60% for HD CD4+ T cells and from 35% to 81% for control CD4+ T cells (225 clones in all). When tested for their ability to respond in autologous mixed lymphocyte reaction (AMLR) against autologous mitomycin-treated non-T cells, 47 of 104 HD clones displayed strong proliferation (SI > 10), whereas none of the 234 CD4+ T cell clones established from normal lymphoid tissues or PB exhibited a proliferative response in AMLR.

The clones proliferated to autologous non-T cells in xenogeneic as well as autologous or allogeneic AB serum (data not shown), thus making it unlikely that any serum component was being recognized in conjunction with self class II molecules. In two cases (LN 110 and LN 115), T cells were also cloned directly in IL-2 under these conditions only T cells that express receptors for IL-2 would undergo clonal expansion. A total of ten CD4+ clones were obtained, and three of them were found to be responsive in AMLR (Table 1), which suggests that part of autoreactive clones were activated in vivo. Taken together, these data suggest that T cell clones derived from lymphoid organs involved by HD respond at a much higher frequency to autologous stimulators. Another possibility is that HD cells are more effective than normal stimulator cells at inducing proliferation in autologous T cell clones. The latter possibility, however, is unlikely on the basis of two additional findings. First, stimulators from two HD patients were unable to induce proliferation in any of 57 T cell clones derived from the PB of a normal individual showing an identical DR phenotype. Second, a cell line derived from T blasts of an AMLR of this normal donor did not show a higher degree of proliferation when tested against stimulators from the two DR-identical HD patients in comparison with autologous stimulators (data not shown).

Part of the T cell clones (77 from four HD patients and 178 from six controls) responding in AMLR were also examined for their ability to proliferate in allogeneic MLR (allo-MLR). To this end, clones were tested against a panel of three EBV-induced B lymphoblastoid cell lines previously screened for their DR haplotype. Among HD patients, a proportion of T cell clones ranging from 19% to 64% (virtually all those responding in AMLR) were found to exhibit consistent proliferation in response to at least one allogeneic stimulator, and the great majority of them responded to more than one DR phenotype. In contrast, the proportion of control clones tested in allo-MLR that responded to at least one DR phenotype was much lower (range, 1.8% to 5.5%), and none of the control clones was found to respond to more than one DR allele. HD T cell clones showing no autoreactivity displayed a pattern of alloreactivity similar to that of control clones. The response to three different allogeneic stimulators of some representative autoreactive HD clones and some alloreactive control clones is reported in Table 2.

In both the AMLR and the allo-MLR the T cells have been shown to proliferate in response to stimulation by products closely linked to class II genes of the major histocompatibility complex (MHC). To provide further evi-
Table 2. Proliferative Response of CD4+ T Cell Clones From HD Patients to Autologous as Well as Different Allogeneic B Lymphoblastoid Cell Lines

<table>
<thead>
<tr>
<th>T Cell Clones Derived From</th>
<th>Medium</th>
<th>Autologous EBV Line</th>
<th>EBV Line PP (DR 1, 5)</th>
<th>EBV Line MB (DR 2, 7)</th>
<th>EBV Line LE (DR 4, 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN 87-4 (DR5, 7)</td>
<td>57</td>
<td>6,351</td>
<td>518</td>
<td>4,210</td>
<td>6,100</td>
</tr>
<tr>
<td>LN 87-8 (DR5, 7)</td>
<td>144</td>
<td>7,940</td>
<td>6,300</td>
<td>4,850</td>
<td>1,780</td>
</tr>
<tr>
<td>LN 87-12 (DR5, 7)</td>
<td>101</td>
<td>5,131</td>
<td>11,280</td>
<td>5,635</td>
<td>4,197</td>
</tr>
<tr>
<td>LN 110-3 (DR2, 5)†</td>
<td>68</td>
<td>13,745</td>
<td>4,280</td>
<td>3,865</td>
<td>ND</td>
</tr>
<tr>
<td>LN 115-21 (DR3, 6)</td>
<td>75</td>
<td>8,045</td>
<td>7,240</td>
<td>6,890</td>
<td>4,566</td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN 26-7 (DR2, 7)</td>
<td>59</td>
<td>112</td>
<td>5,780</td>
<td>84</td>
<td>3,135</td>
</tr>
<tr>
<td>LN 116-34 (DR1, 5)</td>
<td>128</td>
<td>204</td>
<td>309</td>
<td>3,895</td>
<td>235</td>
</tr>
<tr>
<td>LN 116-39 (DR1, 5)</td>
<td>136</td>
<td>106</td>
<td>408</td>
<td>365</td>
<td>7,893</td>
</tr>
<tr>
<td>PB 25-8 (DR2, 5)</td>
<td>100</td>
<td>186</td>
<td>7,209</td>
<td>415</td>
<td>375</td>
</tr>
<tr>
<td>PB 25-11 (DR2, 5)</td>
<td>60</td>
<td>126</td>
<td>330</td>
<td>238</td>
<td>5,924</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Mean values of triplicate determinations. Mean values of counts per minute by cultures containing mitomycin-treated B cells alone were consistently lower than 200.
†LN 110-3 is derived by using IL-2 (see Materials and Methods).

dence that products of MHC class II genes were responsible for the stimulation of AMLR and allo-MLR by T cell clones established from lymphoid organs involved by HD, two T cell clones (LN 115-2 and LN 115-7) were cultured with mitomycin-treated autologous or allogeneic non-T cells in the presence of different concentrations of MoAbs directed against MHC class I or class II (DR) antigens, and their proliferative response to autologous and allogeneic non-T cells was evaluated. The results obtained with the clone LN 115-2 are depicted in Fig 1. The presence of a MoAb reactive with DR antigens in the cultures resulted in a marked reduction in the proliferative response in both AMLR and allo-MLR, whereas the presence of an anti–MHC class I MoAb in the culture did not significantly influence the proliferative response in either AMLR or allo-MLR. Similar results were obtained with the other clone. The inhibitory influence of antibodies in culture could not be attributed to antibody-mediated cellular cytotoxicity. First, the presence in the cultures of anti–MHC class II or class I MoAbs did not inhibit the proliferative response of the same T cell clones to IL-2. In addition, F(ab')2 fragments of anti-DR antibodies were as effective as undigested antibody molecules in inhibiting the proliferative responses (data not shown). Taken together, these data suggest that lymphoid organs involved by HD contain unusually high proportions of T cell clones—part of which were activated in vivo—that respond to both autologous and allogeneic MHC class II products.

To date, no evidence has been provided to suggest an identity between alloreactive T cell clones and clones reactive with autologous determinants of the MHC in the absence of exogenous antigen. The results of this study demonstrate that at least in certain pathological conditions autoreactivity and alloreactivity can coexist in the same T cells. In addition, most T cell clones established from lymphoid organs involved by HD were shown to recognize more than one haplotype. One may suggest that cells capable of responding in both AMLR and allo-MLR were not clonal populations; however, in this experimental system only colonies having a >90% probability of true clonality by Poisson statistics were considered (see Materials and Methods). Moreover, analysis of T cell receptor γ and β gene rearrangements performed on four autoreactive clones showed a rearrangement pattern compatible with clonality and peculiar for each population (data not shown). The latter finding is consistent with recent studies performed on whole cell suspensions that demonstrate polyclonality of T lymphocytes from lymphoid organs involved by HD.

Thus, two hypotheses can be formulated to account for the coexistence of autoreactivity and alloreactivity in the same T cells: the development in HD of T cells with apparent multiple specificity or the emergence in this disease of T cell clones recognizing a common determinant (“public” epitope) of a class II (or related) antigen shared by different human non-T cells or B cell lines. The latter hypothesis is consistent with previous results showing the presence of antibodies against nonpolymorphic determinants of MHC.
class II antigens in the serum of some patients with active HD. The reason for the abnormal recognition of class II determinants by a rather high number of CD4+ T cells in lymphoid organs involved by HD is unclear at the present time. The appearance of T cells with apparent polyclonal anti-Ia specificity, including autoreactivity, has been associated with the development of a graft-vs-host disease-like syndrome in cyclosporine-treated rats after syngeneic bone marrow transplantation. Furthermore, it has been shown that normal MHC class II molecules, including self, trigger the blastogenesis of human T leukemia virus–infected T cells, thus indicating that immunologic recognition of self may be altered by some viruses.

Whatever mechanism is responsible, the presence of a noticeable fraction of T cells showing abnormal recognition of MHC molecules at the site of the lesion in HD supports the view that they may be involved in the pathogenetic mechanisms occurring in this disease. In this regard, it is noteworthy that the neoplastic R-S cell shows a surface membrane antigen (Ki-1) that is preferentially expressed by T blasts from both autologous and allogeneic MLR. Thus, it is tempting to speculate that altered recognition by T cells of self class II antigens is the underlying immunoregulatory dysfunction in HD and that malignant transformation may occur as a final result of this abnormal T cell stimulation.

REFERENCES


High numbers of CD4+ T cells showing abnormal recognition of DR antigens in lymphoid organs involved by Hodgkin's disease

E Maggi, P Parronchi, D Macchia, G Bellesi and S Romagnani