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Cytotoxic T Cells Directed Against HLA-DR Antigens and Their Surface Proteins

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The authors review their recent research involving the generation of cytotoxic T lymphocytes (CTL) directed against HLA-DR antigens. A mouse anti-human xenogeneic system first suggested that HLA-DR antigens could be recognized by CTL. Human allogeneic CTL specific for HLA-DR6 were generated and found to be OKT4$^+$. The fact that these CTL were OKT4$^+$ while anti HLA-A,B CTL were OKT8$^+$ suggested that these T cell surface antigens may be involved in MHC antigen recognition; ie, they may be part of the T cell receptor. These OKT4$^+$, HLA-DR specific CTL were further used to generate monoclonal antibodies (1) which block cytolysis and define novel antigens involved in the CTL-target interaction and (2) which define an antigenic complex on alloantigen activated T cells.

Key words: cytotoxic T cells, HLA-DR antigens, monoclonal antibodies, OKT4, OKT8, OKT3

INTRODUCTION

The considerable number of cell surface molecules that appear to mediate human T-cell functions still require extensive further investigation before we can confidently regard them as clearly identified, adequately characterized, and more explicitly related to demonstrated cell function. In this article, we will attempt to provide an overview of the various results obtained in the course of our recent research conducted in this area of molecular immunology at the Dana-Farber Cancer Institute. Portions of this work have already appeared in the journals cited; other findings represent new information in the fields of immunochemistry and immunobiology. Only reference to our own work is provided in this account. A more complete list of relevant references, including those to the work of other investigators in the field, can be found in the papers cited in this overview.

HLA-DR DIRECTED XENOGENEIC CTL

Five years ago HLA-A,B,C antigens were first inserted into liposomes [1]. These liposomes could be shown to float in a density gradient and to contain the HLA antigens, evident as external "knobs" on vesicles visualized by means of electron microscopy. These purified human antigens within liposomes were subsequently used to stimulate cytotoxic T lymphocytes (CTL) in a secondary stimulation of primed mouse spleen cells [2]. Biochemical analysis, as well as electron microscopy, suggested that virtually all of the antigen was oriented as it was in the cell, ie, with large extracellular domains facing outward. The purified antigens in liposomes were found to be approximately one half as effective as purified cell membranes in stimulating the generation of xenogeneic CTL specific for the HLA-A,B antigens in this secondary response.

Surprisingly, when the proteins of detergent solubilized membranes were examined, removal of the HLA-A,B,C antigens by passage over β_2-microglobulin columns twice did not remove all the CTL-stimulating activity. A residual level of activity remained that could subsequently be absorbed on a lentil lectin column and the eluate from this column, when incorporated into liposomes, was active in stimulating xenogeneic CTL [3]. This eluate was shown to contain HLA-DR antigens. More recently, the same experiments have been carried out with more highly purified HLA-DR antigens eluted from an anti-HLA-DR monoclonal antibody immunoaffinity column. Thus, HLA-DR antigens in liposomes, as

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well as HLA-A,B,C antigens in liposomes, could be shown to stimulate the generation of mouse CTL.

The specificity of these xenogeneic CTL was examined by employing two human B lymphoblastoid cell lines, Daudi and JY, as targets. The Daudi cell line expresses no HLA-A,B,C antigens, although it expresses HLA-DR antigens. JY is a standard line that expresses both HLA-A,B,C and HLA-DR antigens. CTL generated with HLA-A,B,C antigens within liposomes did not kill the Daudi cell line, but CTL generated with HLA-DR antigens in liposomes did. Both CTL preparations killed the JY cell line. These data indicated that xenogeneic CTL could be generated that were directed against HLA-DR as well as against HLA-A,B,C antigens [4].

HLA-DR DIRECTED ALLOGENEIC CTL

Our next step was to attempt to generate allogeneic CTL. For this purpose, the cells from a human MLC, using peripheral blood lymphocytes (PBL) obtained from a laboratory member as the responder cells and irradiated JY cells as the stimulator, were propagated in culture. T-cell growth factor and periodic stimulation with irradiated JY cells were found to be required to maintain growth in culture; if either was omitted, the cells died [5]. When these lines were tested against the standard JY cells, which express both HLA-A,B,C and HLA-DR antigens and against Daudi cells which express only HLA-DR antigen, initially both targets were killed. However, the HLA-A,B,C specific killers were dominant, and the HLA-DR killers disappeared from the culture in a few weeks.

However, if after the initial stimulation with irradiated JY cells, stimulation was continued with irradiated Daudi cells, then HLA-DR specific CTL persisted in culture, and HLA-A,B,C directed killers disappeared [6]. After 12 weeks these uncloned lines killed Daudi cells at an E/T of one or less. Thus, they were very potent CTL lines.

These anti-Daudi CTL lines were observed to be blocked by anti-HLA-DR monoclonal antibodies but not by anti-HLA-A,B,C monoclonal antibodies. Reciprocally, the anti-HLA-A,B,C CTL lines were blocked by anti-HLA-A,B,C monoclonal antibodies but not by anti-HLA-DR monoclonal antibodies. These lines were allospecific. Only HLA-DRw6$^+$ cells were killed by the long-term lines; the stimulator, Daudi, is HLA-DRw6$^+$. Most interestingly, these long term CTL lines were entirely OKT4$^+$ and OKT3$^+$. No OKT8$^+$ cells were found to be present.

These cell lines were then cloned, and two of the clones obtained were studied in some detail [7]. The B8 clone was a DR6 allospecific CTL clone, ie, it killed only DRw6$^+$ lines. The C6 clone appeared, however, to be directed at another subset of the HLA-DR antigens also present on some HLA-DRw6$^+$ cells. This CTL clone recognized only some HLA-DRw6$^+$ targets, as well as some DR5 and DR3 cell lines. The specific subset recognized by this clone remains under study. Among the possibilities currently under consideration are that it could represent an MT or SB subset.

These clones, like the CTL lines, were also OKT4$^+$ and OKT3$^+$ but OKT8$^-$. Several other laboratories have subsequently made similar observations. It is also possible that there are some HLA-DR directed CTL clones that are OKT8$^+$ and OKT4$^-$ and perhaps some HLA-A,B,C directed CTL clones that are OKT4$^+$ and OKT8$^-$, but they must represent a minority of clones. That should not be surprising because structural studies have indicated that, despite their observed functional differences, HLA-DR and HLA-A,B,C antigens are remarkably similar in structure [8].

The fact that anti-HLA-DR CTL are mainly OKT4$^+$ while anti-HLA-A,B,C CTL are OKT8$^+$ strongly suggests that the OKT4 and OKT8 antigens could be part of the T-cell receptor on the CTL which recognizes the MHC antigen, ie, these antigens are not likely to be related to helper and cytotoxic functions of T cells, as had previously been suggested.

MONOCLONAL ANTIBODIES THAT BLOCK KILLING BY CTL LINES

The next step was to attempt to develop monoclonal antibodies (Mabs) that specifically blocked killing. Mice were immunized with one of the long-term OKT4$^+$ CTL lines. Hybridomas were prepared and screened, not for antibody binding, but for secretion of antibodies that blocked killing. This approach had previously been used to define molecules important in mouse CTL-mediated killing [9]. Antibodies which did not block killing were discarded. Four sets of monoclonal antibodies that specifically blocked killing were obtained [10]. Surprisingly, no OKT3 or OKT4-like monoclonal antibodies were detected. Possibly these are not as readily elicited or as potent in blocking killing as the other antibodies that were detected.

The first group, of which two examples were cloned, were HLA-DR-specific antibodies that recognized the target antigen on the target cell. A second class was very prominent. Twenty-two clones were obtained which recognized a heterodimer of 177,000 and 95,000 daltons. They recognized the human homologue of murine LFA-1 [9]. Mabs to LFA-2 (two clones) reacted with a 50,000 dalton polypeptide. A Mab to LFA-3 (one clone) recog-

nized a 60,000 dalton polypeptide. There are at least four epitopes on the LFA-1 molecule, and antibodies directed against the different epitopes each blocked killing to a different extent (Ware: unpublished data).

A variety of functional studies have been carried out (Krensky: unpublished data). Anti LFA-1, -2, and -3 Mabs all block killing by both $OKT8^+$ cell lines directed against HLA-A,B,C antigens and $OKT4^+$ CTL lines directed against HLA-DR antigens. Anti-LFA-1 also blocked natural killer cells, but neither anti-LFA-2 nor anti-LFA-3 did so.

Anti-LFA-1, LFA-2, and LFA-3 Mabs inhibited proliferation as well as cytotoxicity, either proliferation in an MLC or proliferation induced by phytohemagglutinin (PHA). Anti-LFA-1 and LFA-2 Mabs block by interacting with an antigen on the effector cell. When the CTL were pretreated with either of these antibodies and then the antibody was washed out, inhibition occurred. When targets were similarly pretreated, no inhibition occurred. The reverse was true for anti-LFA-3. Anti-LFA-3 appeared to recognize a molecule on the target cell which is involved in the killing reaction.

Among peripheral blood lymphocytes, LFA-1 is present on both B cells and T cells. Upon FACS analysis, it exhibits a bimodal distribution, ie, there is both weak and strong expression on cells. This profile probably does not correspond to B and T cells because the weak set appears too large in number. By contrast, LFA-2 is completely restricted to sheep red blood cell receptor-positive peripheral and activated T cells, including all CTL lines and clones examined. Both FACS analysis and immunoprecipitation demonstrate that the density of LFA-2 is greatest on PHA blasts > CTL > thymocytes > PBL. In addition, LFA-2 has slightly different molecular weight profiles at different stages of activation and/or differentiation. LFA-3 also occurs on both B and T cells and on two separate populations of PBL that express different amounts of this protein. Moreover, it is known to have a rather wide distribution, being present on endothelial cells, vascular smooth muscle, fibroblasts, granulocytes, and platelets.

To summarize, anti-LFA-1 recognizes a heterodimer and inhibits both CTL and NK cytolysis. It inhibits proliferation to a variety of stimuli. LFA-1 is present on both B and T cells and may play some role in cell adhesion in the killing reaction. Anti-LFA-2 recognizes a 50,000 dalton monomer and inhibits CTL lysis, but not NK lysis. It also inhibits proliferative responses. The antigen is present only on T cells and increases in amount upon activation and thus would be a candidate for the T lymphocyte antigen receptor. LFA-3 is a 60,000 dalton antigen which is present on B cells as well as T cells and appears in the killing reaction to be specific for the target cell.

ADDITIONAL MONOCLONAL ANTIBODIES THAT RECOGNIZE CELL SURFACE PROTEINS OF ACTIVATED T CELLS

One other interesting antigenic complex has been recognized by a monoclonal antibody prepared to react with alloantigen activated T cells (Hemler: unpublished data). This antibody, called A-1A5, does not block any functions of T cells. However, in view of the experience with the blocking of function by antibodies directed against different epitopes on molecules, additional examples of monoclonal antibodies corresponding to A-1A5 are being sought in the hope that they may assist in obtaining some clues to its function.

A-1A5 immunoprecipitates a protein triplet of 210, 165, and 135,000 daltons from PHA-activated T cells. It is also present on all CTL lines and clones examined, including both $OKT4^+$ and $OKT8^+$ CTL. It is an especially prominent protein complex on long-term CTL lines and clones.

Peripheral blood lymphocytes, both B and T lymphocytes, are also positive, but they contain only the lowest component of the triplet. The two higher components appear only upon activation of T cells and they are absolutely T cell specific. By simultaneously measuring DNA content and immunofluorescence, A-1A5 was found to be quantitatively increased after cell division, ie, it is activated late after stimulation. The late T-cell activation antigens are believed to be associated with functions of T cells [11]. After growth in the presence of exogenous TCGF, the two upper components become especially prominent.

As already stated, all peripheral T cells were reactive with A-1A5 as were all of a large number of T-cell lines examined. However, like peripheral cells, most of these lines express only the lower component of the triplet. Only one established T-cell line, HSB, is so far known to express any other component of the triplet. This cell line expresses the middle, but not the highest, component of the triplet in addition to the lowest component. B-cell lines express a very small amount of A-1A5 antigen, perhaps one-tenth the amount found on T-cell lines, but again it is only the lower component. A macrophage line, U937, reactive with A-1A5, also expresses the lower component, but its molecular weight is slightly higher than that found on T-cell lines. We do not know if that is a post-translational glycosylation difference or if it represents a different cross-reactive protein. At least one of the components of the protein triplet is a glycoprotein because the complex binds to the lectin, ricin. It can be labeled either by ^{125}I or by [^{35}S] methionine. All three subunits of the complex have internal disulphides because their gel mobilities change after reduction.

Two more monoclonal antibodies that recognize the A-1A5-reactive complex have recently been obtained. One of these (TS2/16) has the same activity as A-1A5, but the second (TS2/7) apparently recognizes a subset of the complex which includes the 210 kd upper band and the 135 kd lower band of the triplet. This new monoclonal antibody permitted us to carry out experiments that showed that this triplet is in fact made up of two heterodimers, which share a small subunit (Hemler: unpublished data). One of the heterodimers ($\alpha^1\beta$) contains the 210 kd upper band plus the 135 kd smallest band while the other ($\alpha^2\beta$) contains the 165 kd middle band plus the 135 kd smallest band. Additional monoclonal antibodies to these polypeptides are being generated to see if some that will uniquely recognize the $\alpha^2\beta$ heterodimer can be obtained as well as to examine the blocking of T-lymphocyte functions by a larger number of Mabs.

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REFERENCES

1. Engelhard VH, Guild BC, Helenius A, Terhorst C, Strominger JL: Reconstitution of purified detergent-soluble HLA-A and HLA-B antigens into phospholipid vesicles. Proc Natl Acad Sci USA 75:3230–3234, 1978.
2. Engelhard VH, Strominger JL, Mescher M, Burakoff SJ: Induction of secondary cytotoxic T lymphocytes by purified HLA-A antigens and HLA-B antigens reconstituted into phospholipid vesicles. Proc Natl Acad Sci USA 75:5688–5691, 1978.
3. Burakoff SJ, Engelhard VH, Kaufman J, Strominger JL: Induction of secondary cytotoxic T lymphocytes by liposomes containing HLA-DR antigens. Nature 283:495–497, 1980.
4. Engelhard VH, Kaufman J, Strominger JL, Burakoff SJ: Specificity of mouse cytotoxic T lymphocytes stimulated with either HLA-A and -B or HLA-DR antigens reconstituted into phospholipid vesicles. J Exp Med 152:54s–64s, 1980.
5. Reiss CS, Hemler ME, Engelhard VH, Mier JW, Strominger JL, Burakoff SJ: Development and characterization of allospecific long-term human cytolytic T-cell lines. Proc Natl Acad Sci USA 77:5432–5436, 1980.
6. Krensky AM, Reiss CA, Mier JW, Strominger JL, Burakoff SJ: Long-term human cytolytic T-cell lines allospecific for HLA-DR6 antigen are $OKT4^+$. Proc Natl Acad Sci USA 79:2365–2369, 1982.
7. Krensky AM, Clayberger C, Reiss CS, Strominger JL, Burakoff SJ: Specificity of $OKT4^+$ cytotoxic lymphocyte clones. J Immunol 129:2001–2003, 1982.
8. Shackelford DA, Kaufman JF, Korman AJ, Strominger JL: HLA-DR antigens: Structure, separation of subpopulations, gene cloning and function. Immunol Rev 66:133–187, 1982.
9. Springer TA, Davignon D, Ho M-K, Kurzinger K, Martz E, Sanchez-Madrid F: LFA-1 and LYT2,3 molecules associated with lymphocyte-T-mediated killing and MAC-1 and LFA-1 homolog associated with complement receptor function. Immunol Rev 68:171–195, 1982.
10. Sanchez-Madrid F, Krensky AM, Ware CF, Robbins E, Strominger JL, Burakoff SJ, Springer TA: Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc Natl Acad Sci USA 79:7489–7493, 1982.
11. Cotner T, Williams JM, Christenson L, Shapiro H, Strom TB, Strominger JL: Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. J Exp Med 157:461–472, 1983.