CLONAL DISTRIBUTION OF HLA-RESTRICTED ANTIGEN-REACTIVE T CELLS IN MAN*

By H. HIRSCHBERG,‡ O. J. BERGH, AND E. THORSBY

From the Tissue Typing Laboratory, Rikshospitalet, The National Hospital, Oslo 1, Norway

In humans, gene products of the major histocompatibility complex (HLA) are involved in the interaction of T cells and monocytes/macrophages in immune responses to antigen (1-3). In particular, in vitro activation of T cells from in vivo sensitized individuals requires corecognition of HLA-D/DR or related structures on monocytes/macrophages, together with antigen, for optimal response (1, 4). This restriction phenomenon has been accounted for by postulating that either T lymphocytes have receptors for new antigenic determinants formed by foreign antigen complexed with self-HLA products (altered self hypothesis) or that T cells have two receptors, one for self-HLA determinants and one for foreign antigen (dual recognition hypothesis). Previous studies with F1 hybrids from inbred strains of guinea pigs have demonstrated two independent subpopulations of antigen-reactive T lymphocytes in these animals, capable of being activated by antigen-pulsed macrophages from either parental strain (5). We report here the results of experiments using the [3H]-thymidine suicide technique (6) indicating that the response of immune human T cells from HLA-D/DR heterozygous donors to the soluble protein antigen purified protein derivative (PPD) is clonally expressed and consists of the concurrent proliferation of at least two separable subpopulations of cells. In addition, using in vitro priming techniques (7, 8) of in vivo sensitized lymphocytes from heterozygous donors, it was possible to generate specific memory cells capable of recognizing the priming soluble protein antigen together with the HLA-D determinant present in the initial sensitizing culture. The use of unrelated monocyte donors demonstrates the importance of the HLA-D/DR antigens for T-cell-monocyte cooperation.

Materials and Methods

Peripheral blood mononuclear cells were isolated by conventional techniques from previously BCG-vaccinated healthy donors who had demonstrated a primary in vitro proliferative response to PPD. T lymphocytes and non-T cells were fractionated using AET-sheep erythrocyte rosette formation followed by Ficoll-Isopaque (Lymphoprep, Nyegaard & Co., AS, Oslo, Norway) flotation. This was followed by a 2-h incubation in plastic tissue culture flasks, removing the majority of adherent cells. T lymphocytes purified in this manner did not respond to PPD in the concentrations used in this study, and contained <1% latex-ingesting cells. In all of the experiments performed, the non-T-cell fraction, consisting of ~50% monocytes (determined by latex ingestion) and contaminating B cells were used directly, without further purification. Because B cells have been shown incapable of cooperating with T cells in a proliferative response to PPD, the monocytes in the non-T-cell fraction are the functional cells (9). The monocytes were irradiated with 25 Gy before use in all cases.

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‡ Present address: Department of Neurosurgery, Rikshospitalet, The National Hospital, Oslo 1, Norway.
The ³H-thymidine suicide technique was performed by incubating 5 × 10⁶ purified T cells with 1 × 10⁶ monocytes irradiated with 25 Gy in tissue culture tubes together with 2.5 μg/ml PPD in 5 ml RPMI-1640 augmented with antibiotics and 20% normal human serum. 5 × 10³ irradiated monocytes were distributed into the wells of flat-bottomed microplates in a vol of 100 μl tissue culture medium and incubated at 37°C until required. After 24 and 48 h of incubation, 10 μCi of ³H-thymidine, sp act 40–60 Ci/mmol, was added to each tube. 24 h later, the cells were washed twice, resuspended, and 50-μl aliquots containing 5 × 10⁴ cells were redistributed into the wells of the prepared flat-bottomed microplates containing the irradiated monocytes. 2.5 μg/ml PPD in 50-μl vol was added to the appropriate cultures. The plates were incubated for an additional 96 h, pulsed with 0.06 μCi [³C]thymidine and harvested 18 h later. The results are given as the median count per minute and range of triplicate cultures.

Monocytes were obtained from either the HLA-D/DR heterozygous responding cell donor or from well characterized, unrelated homozygous cell donors sharing one or the other HLA-D/DR antigen with the T-cell donor. Secondary cultures were obtained by incubating 5 × 10⁸ fractionated T lymphocytes together with monocytes from the T-cell donor or one of a pair of allogeneic homozygous donors and 0.025 μg/ml PPD for 12 d in 5 ml of tissue culture medium. The T-cell fraction of the cultures was reseparated and 5 × 10⁴ T lymphocytes were redistributed in triplicate in the wells of round-bottomed microplates together with PPD with or without 1 × 10⁵ monocytes in 150 μl of tissue culture medium. The plates were incubated for a total of 72 h and harvested after a 6-h pulse with ³H-thymidine.

Results and Discussion

The effects of ³H-thymidine hot pulse treatment on the PPD response of T cells cocultured together with autologous irradiated monocytes during the preincubation period was studied in a series of five experiments. Identical cultures were established and received the same hot pulse treatment, but control cultures contained no antigen during the preculture period. The T cells, after a double wash, were then reexposed to PPD in the presence of either autologous or allogeneic monocytes sharing one or no HLA-D antigens with the T-cell donor as indicated in Fig. 1. As can be seen from the figure, the presence of ³H-thymidine of high specific activity in precultures of T cells together with autologous irradiated monocytes, but without antigen, had little effect on the ability of the cells to respond to PPD in secondary cultures when fresh monocytes were added together with the antigen (Fig. 1, stippled column). In contrast, the response of T cells precultured with autologous monocytes, and ³H-thymidine in the presence of PPD, was significantly impaired in all of the combinations tested, including those containing HLA-D/DR incompatible monocytes. This indicates that the subpopulation of responding T cells activated by PPD and autologous monocytes includes those T cells activated by antigen and monocytes from allogeneic HLA-D/DR compatible homozygous donors. In addition, the fact that it was possible to eliminate the PPD response observed in some combinations of T cells and HLA-D/DR incompatible allogeneic monocytes by hot pulse treatment, suggests that the same subpopulation of T cells also responds to antigen presented by either autologous or HLA-D/DR incompatible allogeneic monocytes. The proliferative response to soluble antigens observed with HLA-D/DR incompatible monocytes (10) then might be a result of cross-reactivity at the level of the T-cell receptor.

The lack of response observed in the treated cultures cannot be a result of priming effects or the generation of specific suppressor cells because cultures of T lymphocytes and autologous monocytes and antigen without hot pulse treatment were always capable of being stimulated by the addition of PPD and fresh monocytes after 3 d of preculture (data not shown).
Figs. 1. PPD response of T lymphocytes precultured with autologous irradiated monocytes in the absence (stippled column) or presence (solid column) of PPD during the initial \[^{3}H\]thymidine hot pulse treatment. The results are given as the median cpm and range of triplicate cultures. The abscissa shows the HLA-D/DR typing results of the various monocyte donors. The T-cell donor was HLA-Dw2/Dw9. The first column shows the nonstimulated autologous controls without PPD.

\[^{3}H\]Thymidine suicide PPD precultures were also established employing T lymphocytes from HLA-D/DR heterozygous donors together with monocytes from one of a pair of allogeneic homozygous donors sharing one or the other HLA-D/DR antigen with the T-cell donor. The results of three experiments are shown in Table I. T cells cocultured with HLA-D/DR homozygous monocytes, PPD, and \[^{3}H\]thymidine, whereas nonresponsive to PPD presented with the monocytes used during the hot pulse preculture did respond to PPD when presented together with either autologous monocytes or monocytes from the other homozygous HLA-D/DR compatible donor. Reciprocal results were obtained by interchanging the monocyte donors (second and third line in each experiment shown in Table I). Similar results were obtained in other experiments using cell donors of other HLA-D/DR specificities.

In further experiments we sensitized T cells from an immunized heterozygous donor in vitro by coculturing the T cells in the presence of autologous monocytes or monocytes from HLA-D/DR compatible homozygous donors together with low concentrations of PPD for 12 d. After this period, the T-cell fraction was reseparated as previously described and the cells rechallenged with fresh irradiated monocytes and PPD for an additional 3 d. Fig. 2 shows the results of one experiment. The primed T cells in the absence of monocytes showed no significant proliferation to PPD.
**Table I**

Elimination of Antigen Reactive Clones by $[^{3}H]$Thymidine Treatment

| $[^{3}H]$Thymidine pre-culture | PPD | Second culture* $\Delta$ cpm |
|-------------------------------|-----|-----------------------------|
| T cells | Non-T cells | | | |
| | | 1/3 | 1/1 | 3/3 |
| Exp. 1.§ | 1/3 | 1/3 | - | ND | 2,816 | 1,691 |
| | 1/1 | + | ND | 23 | 1,804 |
| | 3/3 | + | ND | 1,262 | 0 |
| | 1/1 | 1/1 | 4/4 |
| Exp. 2. | 1/4 | 1/4 | - | 2,008 | 3,836 | 982 |
| | 1/1 | + | 1,328 | 0 | 1,488 |
| | 4/4 | + | 1,009 | 2,245 | 257 |
| | 2/9 | 2/2 | 9/9 |
| Exp. 3 | 2/9 | 2/9 | - | 3,614 | 2,380 | 2,276 |
| | 2/2 | + | 1,086 | 61 | 1,036 |
| | 9/9 | + | 1,578 | 1,149 | 261 |

* Unstimulated control cpm have been subtracted and only $\Delta$cpm are shown. The results are derived from the median cpm of triplicate culture.

§ 2.5 $\mu$g/ml PPD was added together with the monocytes.

**Summary**

Lymphocytes primed for PPD, together with autologous monocytes, could be restimulated by PPD in the presence of either one of a pair of HLA-D/DR compatible homozygous monocytes. In experiments were T lymphocytes were primed together with one or the other compatible homozygous monocyte, the proliferative secondary response was always significantly greater when the same monocyte donor was used in the first as well as the second culture. Therefore, the in vitro sensitized T cells only react significantly in secondary cultures in the presence of the monocytes used for priming. These experiments have been repeated using different cell donors, consistently demonstrating the ability to prime in vitro T cells from sensitized donors in an HLA-D/DR clonally restricted manner.

Taken together, our experiments using human cells agree well with the concept that separate subpopulations (clones) of lymphocytes, responsive to self-HLA-D determinants on monocytes/macrophages and antigen, exist in immunized individuals. It has also recently been reported that MHC (HLA-A,B,C) restricted, T-cell-mediated cytotoxicity in man is clonally expressed (11) adding further evidence to the clonal distribution of HLA-restricted lymphocyte function.

The $[^{3}H]$thymidine suicide technique was used to test the hypothesis that the response of immune human T cells from HLA-D/DR heterozygous donors to the soluble protein antigen purified protein derivative (PPD) is clonally expressed and consists of the concurrent proliferation of at least two separable subpopulations of lymphocytes. The results showed that each of the two subpopulations react to one or the other of the HLA-D/DR antigens presented together with PPD by allogeneic
monocytes. In addition, using in vitro priming techniques of in vivo sensitized lymphocytes from heterozygous donors, it was possible to generate specific memory cells capable of recognizing the priming soluble protein antigen together with the HLA-D/DR determinant present in the initial sensitizing culture.

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