HLA RESTRICTION OF HUMAN CYTOTOXIC T LYMPHOCYTES
SPECIFIC FOR INFLUENZA VIRUS
Poor Recognition of Virus Associated with HLA A2

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Cytotoxic T lymphocytes that are specific for virus antigen expressed at the surface of infected cells, have been demonstrated in mice infected with several viruses (1-6). We have recently described the induction of such cells from human peripheral blood lymphocytes (7). Murine cytotoxic T cells show specificity for both the viral and H-2 antigens on the infected target cell (1-6). Killer and target cells must share H-2 antigens of the K and D series (1-6). Ia antigens do not influence this interaction though Schmidt-Verhulst and Shearer (8) and Gordon and Simpson (9) have shown that I region products can influence the generation of cytotoxic T cells in vitro.

The requirement for H-2 sharing (H-2 restriction) has been shown for several viruses including lymphocytic choriomeningitis virus, vaccinia, ectromelia, influenza, and Friend leukemia virus (1-6). These findings suggest that the natural function of H-2 antigens might be to interact with viruses in the immune response to virus infection. The H-2 restriction phenomenon, however, is not limited to virus infection; it is also seen for cytotoxic cells generated against chemically altered cell surface products (8) or to minor transplantation antigens (9, 10).

Demonstration of HLA restriction of human cytotoxic cell activity has been more difficult, probably because of technical and ethical problems in experimental design. However, HLA restriction has been demonstrated for cytotoxic T-cell lysis of cells differing in minor transplantation antigens (11), of cells that have been dinitrophenylated (12) and of cells infected with influenza viruses (7). This report extends the evidence for HLA antigen restriction in cytotoxicity of influenza virus infected cells. The A and B loci are shown to be involved and it is demonstrated that there is a deficiency in cytotoxic T lymphocyte recognition of influenza virus associated with HLA A2.

Materials and Methods

Lymphocytes. Venous blood was taken from healthy volunteers and mononuclear cells separated on Triosil-Ficoll (Lymphoprep, Nyegaard and Co., Oslo, Norway).

HLA Typing. HLA A B C and DR types were determined using standard techniques (13) by Dr. Philippa Cullen and Dr. Alan Ting of the Nuffield Department of Surgery Tissue Typing Laboratory. Most individuals were typed at the D and DR loci by the mixed lymphocyte culture (MLC)2 and serological methods, respectively. The results using these two

* Supported by a Wellcome Senior Research Fellowship and by a Medical Research Council Programme grant to Professor Morris.

1 A. J. McMichael and B. A. Askonas. 1978. Influenza specific cytotoxic T cells in man; induction and properties of the cytotoxic cell. Eur. J. Immunol. In press.

2 Abbreviations used in this paper: FCS, fetal calf serum; HA, hemagglutinin; hf, haplotype frequency; K: T, ratio, killer cell: target cell ratio; MLC, mixed lymphocyte culture; pbl, peripheral blood lymphocyte.

1458  J. EXP. Med. © The Rockefeller University Press - 0022-1007/78/1201-1458$1.00
Volume 148  December 1978  1458-1467
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No. % Lysis by C.W. HLA Shared

Fig. 1. Lysis of 26 allogeneic influenza virus infected target cells by C.W.: the results of six separate experiments have been combined. In each, C.W. pbl were sensitized to autologous influenza virus A/X31 infected cells and then tested on autologous and allogeneic targets at a K:T ratio of 50:1. The target cells are grouped in the figure according to their degree of HLA antigen sharing with the killer cell C.W. (HLA: AW24, B7, DW1/AW24, B7, DW2), indicated by cross-hatching.

For each group the mean lysis is shown with standard error if there was more than one target in the group. The value for autologous target cell lysis is also the mean value obtained and is shown with its standard error.

techniques were concordant and when both types were determined, for simplicity, only the D type is shown.

Induction of Influenza Virus Specific Cytotoxic Lymphocytes. The sensitization of peripheral blood lymphocytes (pbl) with autologous lymphocytes which have been infected with influenza virus A/X31, has been described in detail previously. In brief, 3-5 × 10⁶ pbl were infected by incubation with 100 hemagglutinin units (HA) of influenza virus A/X31 for 90 min at 37°C in serum-free medium. They were then incubated with 20-40 × 10⁶ autologous pbl in RPMI-1640/10% FCS for 5-8 days at 37°C in 5% CO₂/air.

Chromium ⁵¹ Release Assay. Effector lymphocytes were harvested at the end of the 5-8 day culture period and viability determined. Target cells were prepared from pbl that had been separated from venous blood the previous day. 3 × 10⁶ pbl in 0.1 ml RPMI-1640 were incubated with 100 HA units influenza virus A/X31 and 100 μCi Cr⁵¹ (Sodium chromate, Amersham Corp., Arlington Heights, Ill.) for 90 min. The cells were then washed thrice and incubated in RPMI-1640/10% FCS for an additional 4 h.

10⁶ target cells in 50 μl medium were incubated in duplicate in microtiter trays (Linbro Chemical Co., Hamden, Conn.) with 5 × 10⁶ or 7.5 × 10⁶ viable effector cells also in 50 μl, for 5 h at 37°C. They were then harvested either by pipetting off 50 μl of supernate after centrifuging the tray, or using a Titertek harvester (Flow Laboratories Inc., Rockville, Md.). Low and high controls were target cells incubated in quadruplicate with 50 μl medium and 50 μl 5% Triton X-100, respectively. Results were expressed as specific lysis:

\[
\frac{\text{Cr}^{51} \text{ release by killer cell} - \text{Cr}^{51} \text{ release by medium}}{\text{Cr}^{51} \text{ release by medium}} \times 100.
\]

\[
\text{Cr}^{51} \text{ release by medium was usually less than 20% of Cr}^{51} \text{ release by Triton X-100. Standard error of the mean lysis in each experiment was less than ± 5%.

Results

HLA Loci Involved in the Cytolysis of Influenza Virus Infected Target Cells. It has been shown previously that two individuals C.W. and F.W. showed HLA restriction in the T-cell-mediated lysis of allogeneic virus infected target cells (7). Cytotoxic cells from C.W. have now been tested on 26 different target cell preparations. Fig. 1 summarizes the results of six separate experiments. The target cells are grouped according to the HLA antigens shared. Thus C.W. cytotoxic T cells killed target cells that shared the HLA haplotypes AW24-B7-DW2, B7-DW2, or the HLA antigens AW24 (an antigenic
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Fig. 2. Lysis of 20 allogeneic influenza virus infected target cells by F.W.: see legend to Fig. 1. HLA type of F.W. is A1, B8, DW3/A1, B8, D-.

The D type of many of these target cells was not determined and the effect of D antigen sharing could not be investigated in these experiments.

split of A9) or B7. Influenza virus infected cells that shared only HLA DW2 or DW1 and those that shared no HLA antigens were not lysed.

In similar experiments, F.W. cells lysed target cells that shared HLA A1-B8, A1 or B8 but did not lyse target cells that shared neither of these antigens. Fig. 2. There was insufficient data to determine unequivocably whether D antigen sharing would allow lysis by F.W. but one target cell, L.H., which shared HLA DW3 gave only 0.6% lysis. The HLA restriction phenomenon thus appears to be an HLA A-B locus effect. Because of the extreme polymorphism of the HLA system, it was of interest to determine whether particular HLA antigens would interact in different ways with the influenza virus in determining the function of the cytotoxic T lymphocytes.

Frequent Failure of Target Cell Lysis when HLA A2 is Shared. A2 is the most common HLA antigen in Caucasians (gene frequency 0.3). It was observed that cytotoxic T cells from several individuals did not lyse target cells which shared this antigen (Fig. 3). This was not an absolute deficiency, however, because some cytotoxic cells lysed target cells from one or two individuals that apparently shared only HLA A2.

It was considered possible that the HLA A2 positive target cells could be resistant to infection with influenza A/X31 virus although such an effect would clearly be associated with the presence of the HLA A2 antigen. To exclude this, experiments were set up when the HLA A2 positive target cell was lysed either by its autologous cytotoxic cell or by a killer cell that shared another HLA antigen such as HLA B7. The experiments shown in Fig. 4, show that target cells with the HLA phenotype A2, AX, B7, BY, were lysed by effector cells that shared HLA B7 but not by effector cells that shared only HLA A2. Similarly target cells with the HLA phenotype A2, AX, BY BZ were lysed by autologous cytotoxic cells but not by those sharing HLA A2.

Direct Comparison of Lysis when HLA A2 and B7 are Shared. Cytotoxic lymphocytes were generated from donors that possessed the phenotype HLA A2, B7 and were tested on target cells that shared either HLA A2 or HLA B7. The results shown in Fig. 5 show that lysis was seen when HLA B7 was shared but that the levels of cytotoxicity were, with one exception, very low when only HLA A2 was shared.

Fig. 6 summarizes the results seen in all pairings of HLA A2, B7, and B8. In this figure, the results from 39 separate experiments using effector cells from 15 donors and target cells from 41 donors, are compared. To make the comparison, the data are standardized with the lysis seen for each killer cell target cell pair being expressed as a percentage of that cytotoxic cell's lysis of autologous infected target cells. Several combinations were tested more than once and then a mean value was taken and each
Fig. 3. Lysis of allogeneic influenza virus infected target cells by J.R., A.M., and A.T. See legend to Fig. 1.

pair is expressed as a single point. This method of normalization suffers from the drawback that discrepant values may be generated if the autologous target cell was for some reason substandard and gave an artificially low lysis value; some of the values over 100% may be due to this. As selection of the data, however, could introduce bias, all results were included and it was assumed that the volume of data—150 pairs tested—would minimize such discrepancies. This appears to have happened, (Fig. 6) and when HLA B7 or B8 sharing is compared with no antigen sharing, the results are remarkably consistent with the murine histocompatibility restriction phenomena. The discordant results with HLA A2 sharing are readily apparent, with half the data points falling below 25% and thus being comparable to results seen when no HLA antigens are shared.

Are there Subgroups of HLA A2? Because sharing of HLA A2 between killer and target allowed lysis in some instances but not in others, the possibility that this serologically homogeneous antigen was, in fact, heterogeneous was considered. If this was true, pairs of HLA 2 positive individuals should behave concordantly in reciprocal testing of cytotoxic cell on target cell. The pooled results of several experiments are shown in Table I. A number of pairs were observed when lysis seen was one-way, which virtually excludes the existence of subgroups. It is possible, however, that there could be high (J.M., J.F.) and low (A.M., A.T., D.H., J.R.) responders to HLA A2 plus influenza virus.

Discussion

The results shown here provide further evidence for HLA restriction in the interaction between the human virus specific cytotoxic T cell and virus infected target
Fig. 4. Cytotoxic cell lysis of influenza virus infected target cells with HLA A2: four experiments are shown, A, B, C, and D. In each experiment two cytotoxic cells were tested on autologous influenza virus infected target cells and on allogeneic target cells at a K:T ratio of 50:1. Note that each column represents a killer cell, (indicated underneath), and that columns are paired for each target cell, (indicated above), so that the format is the opposite of that used in Figs. 1-3 and 5. For HLA types of C.W., A.M., J.R., D.H., and A.T. see Figs. 1, 3, and Table I. HLA type of C.H. is A W23, 11, B7, W39.

Fig. 5. Lysis of influenza virus infected target cells by cytotoxic cells with HLA phenotype A2, B7: each cytotoxic cell (indicated above each histogram) was sensitized by incubation with autologous influenza A/X31 virus infected cells and then tested on influenza virus infected autologous target cells (S) and five allogeneic cells at a K:T ratio of 50:1. Under each allogeneic target cell are indicated the HLA antigens it shared with the cytotoxic cell.
Fig. 6. Summary results of 150 allogeneic killer: target cell pair combinations. Each cytotoxic cell was sensitized with autologous influenza virus infected cells and tested on autologous and allogeneic influenza virus infected target cells. Lysis of autologous cells was called 100% and allogeneic cell lysis expressed as a percentage of that value. Each O represents a different pair. The results are grouped according to whether killer and target cell shared: no HLA antigens; HLA B7; HLA B8; or only HLA A2.

cell. Detailed data is provided for two individuals C.W. and F.W., and sharing of HLA AW24, A3, B7, A1, or B8 allows lysis to occur. Although CW and FW are HLA A and B homozygous, the restriction is not limited to HLA homozygotes. Individuals D.H., J.F., B.M., A.M., and C.H. shown in Figs. 3–5 are HLA A and B heterozygous and lysed allogeneic target cells that shared HLA antigens.

These results and the combined data from 15 killer cell populations, demonstrate very clearly that the restriction phenomenon, first described by Zinkernagel and Doherty (5), applies to man at least with respect to HLA, A1, AW24, B7, B8, B27, and BW21. Nonspecific lysis of target cells when no HLA antigen was shared was seen only once, at a marginal level, in 38 combinations. All other exceptions are of a conservative type in that the requirements for identity between killer and target cell seem to be very strict.

Does the fact that this phenomenon can be demonstrated in an outbred species such as man, indicate that it is the HLA antigens themselves and not linked products
Table I
Cross Testing of Influenza Specific HLA A2 Positive Cytotoxic Cells

| Killer cell | HLA A | HLA B | DR | Target cell | A.M. | A.T. | J.M. | N.C. | J.F. | D.H. | J.R. |
|-------------|-------|-------|----|-------------|------|------|------|------|------|------|------|
| A.M.        | 2, W2; | W21, W40; | W4,— |          | 100* (10) | NT   | NT   | 10   | NT   | 24   |
| A.T.        | 2, 11; | W40, 22; | W4,— |          | (11) 100 | 6    | 25   | NT   | 1    | 120  |
| J.M.        | 2, 2;  | 5, W15; | W4,— |          | 35    | NT   | 100  | 78   | 42   | 0    | 150  |
| N.C.        | 2, 3;  | 7, 8;   | NT   |          | NT    | NT   | 72   | 100  | (90) | NT   |      |
| J.F.        | 2, 3;  | 7, 27;  | W4, W2|          | 0     | 57   | 77   | (98) | 100  | (44) | (51) |
| D.H.        | 2, 3;  | 7, ET;  | W1, W7|          | 18    | 10   | 14   | (90) | (105)| 100  | 92   |
| J.R.        | 2, 2;  | 12, 27; | W4, W1|          | 16    | 46   | 10   | NT   | (11) | 7    | 100  |

One way interactions:
A.T. → J.R.
J.M. → J.R.
J.F. → J.M.
D.H. → J.R.

* Lysis of autologous influenza virus infected target cells expressed as 100%.
† Parentheses indicate sharing between killer and target of HLA A or B antigens other than HLA A2.
§ Arrows: A → B indicate that A lyse B but B does not lyse A; broken arrows indicate that lysis in the reverse direction is equivocal (25-50%).

that are involved in this interaction? In many pairs there was an HLA haplotype shared and the antigens were in linkage disequilibrium (14, 15) i.e. B7-D W2, (Δ: 0.039 haplotype frequency (hf) 0.047), B8-DW3 (Δ: 0.044 hf 0.049) A1-B8 (Δ: 0.077 hf 0.098) so that linked products could be involved. There are enough examples of single antigens shared, however, e.g. AW24, B7, A1, B8 to indicate that it is the HLA antigen itself that is involved. This is an important point with respect to studies in the inbred mouse strains when there is almost invariably some degree of H-2 haplotype sharing. It is in accord with the finding of Zinkernagel (16) that mutant H-2 antigens do not interact with the wild type.

The HLA loci involved in this phenomenon are clearly HLA A and HLA B. The data for HLA-C are as yet inadequate, because the blank antigen frequency is too high and it is difficult to find pairs who share only C locus antigens. At first sight the lack of any clear lysis of target cells in the 38 pairs that shared neither A nor B antigens (Fig. 6) would seem to exclude a role for C locus products. C antigens, however, are mostly in strong linkage disequilibrium with B antigens (17) so that it is possible that no C antigens were shared in this group. HLA D antigens in this study were defined by the MLC reaction and serologically. When HLA D antigens were shared lysis was not seen. This is to be expected if HLA-D is equivalent to the mouse H-2 I region, which is not involved in these interactions (1–6). The low lysis observed when an HLA D type was shared between killer and target could, however, only reflect the lack of expression of HLA D antigens in the target cells which are largely T lymphocytes. This possibility seems unlikely but does remain to be excluded.

Having established that the HLA antigens are involved, it is important to determine whether all HLA antigens are equally effective. A striking discrepancy was found for HLA A2. This antigen is the commonest HLA antigen, with a gene frequency in Caucasians of 0.3. It is found in all racial groups and could indeed be closely related to the ancestral A locus HLA antigen. In 68 pairings, in which HLA A2 was the only HLA A or B locus product shared, no lysis was seen in 32 of them, which contrasts
The HLA A2 target cells were expressing influenza antigens, because they were lysed either by autologous killer cells; by those sharing other HLA antigens (Fig. 4); or in a few cases by some killer cells sharing HLA A2. Similarly, killer cells that were HLA A2 positive could lyse infected target cells that shared other HLA antigens (Fig. 5).

Two simple explanations for these findings appear to be excluded. HLA A2 does not appear to be composed of many subgroups of antigens. There is no serological evidence for this and mutually interacting sub groups were not found in these experiments. Second, if the restriction phenomenon is explained by the altered self hypothesis (1) an absolute failure of interaction between HLA A2 and virus as occurs between most H2 K and D products and Friend leukemia virus (18) is excluded by the occasions when HLA A2 sharing has allowed lysis.

The explanation must therefore lie in the influence of other factors. It may not be necessary to look beyond other HLA products. Gordon and Simpson (9) and Schmidt-Verhulst and Shearer (8) have demonstrated that Ir genes regulate recognition of target cells by cytotoxic cells. It is possible therefore, that in some individuals the Ir genes involved in the recognition of HLA A2 and influenza virus may be of the low response type. The data shown in Table I, although somewhat preliminary do suggest that there could be high and low responders with respect to lysis of target cells sharing HLA A2 and influenza virus. The DR (Ia) types of the high and low responders to HLA A2-influenza virus have been compared and no obvious DR specificity stands out. Nevertheless, Ia positive cells are clearly involved in the generation of the cytotoxic cell and this remains a possibility.1

A more plausible hypothesis is that, of the HLA A, B (and C ?) products at the cell surface, some antigens may dominate. This could either be in direct association with the virus protein or in the interaction with the cytotoxic cell. HLA A2 would thus be dominated by B7 in most circumstances. The interaction between a mosaic of cell surface HLA antigens and viral antigens could be very complex. Some evidence for this comes from the results of Gordon et al. (19). They found that in the cytolysis of Y antigen bearing target cells that an Fl cytotoxic cell, sensitized with an Fl male cell, lysed preferentially parental cells of one haplotype.

An important question which remains to be resolved, is whether the poor recognition of influenza virus infected target cells by killer cells that share only HLA A2 is specific for influenza antigens. Preliminary results using the influenza virus Hongkong B show that the results were very similar to those obtained with influenza A/X31. Goulmy et al. (11) and Dickmeiss et al. (12), however, have shown that in killing of target cells by CTL sensitised to a male transplantation antigen and DNP, respectively, HLA A2 sharing was particularly effective in allowing lysis. These results therefore strongly suggest that HLA A2 functions differently for these three very disparate antigens.

The findings that certain HLA antigens allow cytolysis when shared and others do not, has important implications. It is well known that HLA antigens can confer disease susceptibility (20, 21). The failure of certain antigens to interact with a virus to provoke a cytotoxic immune response, could explain some of these associations where HLA A, B or C antigens are primarily involved. Piazza et al. (22) have demonstrated that HLA A or B antigens could be involved in resistance to malaria and de Vries et al. (23) have similar data for leprosy. Otherwise, few studies of disease association with HLA have addressed the possibility that the HLA antigens could
confer resistance to infectious disease. This study suggests that particular antigens could be advantageous in certain virus diseases. If each HLA antigen interacts with a distinct group of specific viruses, individuals heterozygous at duplicated HLA loci could have a selective advantage and thus the HLA polymorphism could be generated and maintained.

Summary

Cytotoxic T lymphocytes (CTL), specific for influenza A/X31 virus, were generated from human peripheral blood lymphocytes. These CTL lysed target cells that were infected with the same virus and that shared HLA A or B locus antigens. Minimal lysis was observed when HLA-D antigens were shared. Not all HLA A and B antigens were equally effective. Efficient lysis of target cells was seen when HLA A1, A3, B7, B8, B27 and BW21 were shared with the CTL, but when HLA A2 was the only shared antigen lysis was usually minimal. This deficiency in CTL function associated with HLA A2 was not absolute. It is suggested that the function of this antigen might be influenced by other surface molecules on the cell and in particular the other HLA products.

I would like to thank Ms. Jenny Pilch for expert technical assistance; Dr. John Skehel for supplying the influenza virus, and Dr. Ita Askonas and Professor Peter Morris for advice and encouragement. I am also indebted to all those who have volunteered to give blood samples and to Dr. Philippa Cullen and Dr. Alan Ting for HLA typing them.

Received for publication 10 July 1978.

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