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The proliferative T cell response to herpes simplex virus (HSV) antigen is restricted by self HLA-D

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SUMMARY

T cells from sensitized individuals react with a proliferative response to herpes simplex virus antigen (HSV-Ag) *in vitro*, when antigen is presented together with autologous macrophages (Mø). We here report that this T-Mø co-operation is restricted by self HLA-D/DR molecules of the sensitized T cell donor. Furthermore, it is shown that the lack of co-operation between T cells and HLA-D/DR disparate macrophages, is due neither to suppression nor to generation of cytotoxic T cells in the allogeneic mixture.

INTRODUCTION

The *in vitro* response to herpes simplex virus antigen (HSV-Ag) in man is T cell-mediated requiring pre-sensitization of the T cell donor as well as co-operation from autologous macrophages. In a previous article (Berle & Thorsby, 1979), we reported that only T cells from cell donors sensitized against herpes simplex virus type I (i.e. clinical history of recurrent herpes labialis and increased anti-herpes neutralizing antibodies) will mount an HSV-Ag specific response *in vitro* and that this T cell response takes place only in the presence of macrophages functioning as antigen-presenting cells. In contrast, T cells from neonates and other non-sensitized individuals do not respond to HSV-Ag, even if autologous macrophages are present.

Bergholtz & Thorsby (1977, 1978) have shown that the macrophage-dependent proliferative T cell response to antigens such as PPD is restricted by self-HLA-D/DR molecules of the T cell donor. HLA-D restriction has been found also using the hapten TNP (Seldin & Rich, 1978; Thorsby & Nousiainen, 1979). We were interested to find out whether the proliferative T cell response to certain virus antigens showed a similar restriction. For this purpose we have studied the proliferative response *in vitro* to HSV-Ag in various HLA-D/DR compatible and incompatible combinations of T cells and macrophages. In this article we report that the HSV-Ag-specific, macrophage-dependent T cell response, is restricted by the self-HLA-D/DR molecules of the T cell donor. The response requires sharing of at least one HLA-D/DR antigen between T cell and macrophage donor. The macrophages, however, may originate from donors who are not sensitized to HSV. We report further that macrophages not sharing any HLA-D/DR antigens with the T cell donor are incapable of functioning as antigen presenting cells. This lack of co-operation is shown not to be due to suppression or to generation of cytotoxicity.

MATERIALS AND METHODS

Cell donors. Blood samples were taken from otherwise healthy members of the hospital staff. The cell donors were grouped according to the presence or absence of recurrent herpes labialis (based on clinical evidence and/or additional isolation of HSV-type I). Where the clinical history was uncertain a titre of neutralizing anti-herpes simplex virus antibodies of more

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than 1:2 (laboratory standard—Institute of Bacteriology, The National Hospital Oslo) was considered indicative of sensitization against herpes simplex virus type I. Detection of anti-herpes simplex antibodies (neutralization test) was performed by Dr M. Degré Institute of Bacteriology, The National Hospital, Oslo.

Herpes simplex virus antigen. We have used lyophilized herpes simplex virus type I complement fixation antigen produced in monkey kidney cells (Orion Diagnostica, Helsinki, Batch D 1, Cat. no. D 428). The control antigen used (Orion Diagnostica, Batch D 1, Cat. no. D 711) has been processed corresponding to the CF antigen but does not contain herpes simplex virus type I antigen.

HLA-A, -B and -C typing. The typing was performed with a microcomplement-dependent cytotoxicity (CDC) test as described previously (Albrechtsen *et al.*, 1978) using fifty-four highly selected antisera.

HLA-D/DR typing. HLA-D typing was performed with the HCT-technique using homozygous typing cells (Kaakinen *et al.*, 1977) and the serological HLA-DR typing was performed using a complement-dependent cytotoxicity test on purified B cells as described previously (Albrechtsen *et al.*, 1978),

Cell separation technique. Defibrinated blood was separated by the Ficoll–Isopaque flotation technique (Bøyum, 1968), using lymphoprep (Nyco, Oslo, Norway). The PBM thus obtained were washed twice at 600 *g* in RPMI 1640 (GIBCO-Biocult, Glasgow, Scotland).

T cell preparion. PBM were suspended in tissue culture medium, i.e. RPMI 1640 with 100 i.u. penicillin/ml, 100 μg/ml streptomycin and 20% normal serum from a pool of healthy blood donors. (This serum was not screened for anti-herpes virus antibodies.) The cells were incubated in 25 cm^2 flat-bottomed tissue culture flasks (Cat. no. 3013, Falcon, California, USA) for 2 hr at 37°C. Following a 2-hr incubation, the non-adherent cells were pipetted off and made to form rosettes with AET-treated sheep red blood cells (SRBC) (Pellegrino, Ferrone & Theophilopoulus, 1976). The rosettes were gently resuspended in RPMI 1640 without serum and separated from 'non-T' cells by Ficoll–Isopaque flotation. The SRBC were lysed through incubation in normal serum for 20 min at 37°C, washed twice and resuspended in tissue culture medium. The cell suspension thus obtained was incubated in flat-bottomed tissue culture flasks overnight at 37°C in order to remove further remaining adherent cells. After approximately 20 hr of incubation, the non-adherent cells were pipetted off, washed twice in RPMI 1640 at 600 *g* and resuspended in tissue culture medium, and were again made to form rosettes with AET-treated SRBC. The non-rosette forming cells were removed after Ficoll–Isopaque flotation. The SRBC were lysed with normal serum. The cells were washed twice, resuspended in tissue culture medium and adjusted to 1×10^6 cells per ml. The resulting cell population was designated 'T cells'. Among these more than 80% formed rosettes with AET-treated SRBC, less than 2% ingested latex. Less than 5% were Ig positive cells and 95% proved viable by means of trypan blue exclusion.

Preparation of Mø. The adherent cells from the PBM and the non-T fraction were incubated overnight in flat-bottomed tissue culture flasks after the non-adherent cells had been removed. After approximately 20 hr of incubation EDTA, 3·3 μg/ml, was added and the cells loosened through vigorous pipetting after incubation on ice for 90 min. The cells were washed three times at 4°C and 500 *g*. After resuspension in tissue culture medium the cells were adjusted to 1×10^5 per ml and kept on ice until used. These adherent cells were designated macrophages (Mø), and contained more than 80% latex ingesting cells. All Mø suspensions were irradiated with 2000 rad before use.

Cell culture techniques. For all experiments, flat-bottomed microtitre plates were used (TC disc 3590, Costar, USA). To study whether soluble HSV-Ag added directly to the T-macrophage culture or pulsation of the macrophages, i.e. pre-incubation with the same antigen, yield any difference, we followed two different approaches:

(1) Soluble HSV-Ag was added directly to the T-macrophage mixtures, using 50,000 T cells and 5,000 macrophages per well in a total tissue culture medium volume of 170 μl. The final antigen concentration was kept at 1:100, based on our previously reported dose–response investigations (Berle & Thorsby, 1979).

(2) Five thousand macrophages per well were incubated in 170 μl tissue culture medium with a final concentration of HSV-Ag 1:100. After approximately 20 hr of incubation with antigen, the macrophages were washed twice directly in the plate, using 150 μl RPMI 1640 per well per washing. Thereafter, 50,000 T cells were added to each well.

We also performed dose–response studies using a constant number of T cells and macrophages in decreasing concentrations.

All combinations were performed in triplicate and cultured for 6 days, again based on our previously reported kinetics experiments (Berle & Thorsby, 1979). Following 5 days in culture, 0·2 ml 1·0 μCi of ^{3}H-thymidine was added to each well. After an additional 24 hr of culture, the plates were harvested with a semi-automatic multiple harvester (Skatron, Lierbyen, Norway). Incorporation of ^{3}H-thymidine was assessed by liquid scintillation counting and the results expressed as mean c.p.m.$\pm$s.e. or as median c.p.m. As HSV-Ag specific response, we used the incremental c.p.m. values after subtraction of the response of T cells with macrophages alone and without antigen. The HSV-Ag response was also expressed as per cent relative antigen stimulation (% RAgS) value, where the incremental response with allogeneic macrophages was expressed as a percentage of the response with autologous antigen presenting cells (macrophages).

RESULTS

Presensitization of the cell donors

Knowing that the T cells had to originate from an HSV-sensitized cell donor in order to proliferate in the presence of HSV-Ag and macrophages, we first investigated whether the macrophages also had to

originate from HSV-sensitized donors. The results from one out of three experiments are shown in Table 1. Cells were taken from unrelated HLA-D/DR identical individuals, A and B. Of these, A presented clinical evidence of recurrent herpes labialis, and had HSV virus type I isolated from a perioral blister during one attack. Cell donor B presented no such history. It can be seen that only in T cell donor A a macrophage dependent proliferative T cell response is found, reflecting the donor's state of HSV sensitization. Cell donor B does not show a T cell response to HSV-Ag. The macrophages from this non-sensitized donor, however, are capable of reconstituting the macrophage-dependent T cell response of the sensitized T cell donor A. Whereas cell donor B did not respond to the HSV-Ag, the proliferative capability of his T cells was ascertained in MLC (results not shown).

TABLE 1. HSV-Ag-specific T cell response is macrophage-dependent and requires sensitized T cells

T cell* donor	HSV-Ag†		Macrophages‡ A	Macrophages‡ B
A	+	824	14,496	11,774
Herpes+	−	226	201	618
DR 1/7	Δ§	598	14,295	11,156
B	+	546	1072	557
Herpes−	−	517	479	1047
DR 1/7	Δ	29	593	—

* Presence (+) or absence (−) of clinical history of recurrent herpes labialis. 5×10^4 T cells prepared as described in the Materials and Methods section.

† Final HSV-Ag concentration 1:100.

‡ 5×10^3 macrophages prepared as described in the Materials and Methods section.

§ Incremental c.p.m.

TABLE 2. HLA-D restriction of T cell response to HSV-Ag

HSV-Ag*	T cells† alone	T cells+Mø* autologous	T cells+Mø* allogeneic			
		DR 1/8	DR 1/8	DR 1/7	DR 3/8	DR 2/6
+	847±35‡	10,040±1901	9671±1380	9407±911	10,400±1265	3345±631
−	1120±208	1129±362	955±101	1206±32	1733±272	1824±154
Δ§		8911	8716	8201	8667	1621
		DR 2/5	DR 2/5	DR 2/−	DR 5/8	DR 1/8
+	70±23	7631±172	6724±942	6507±523	7178±1204	11,995±1497
−	174±36	99±7	386±94	2133±125	1975±619	10,204±1360
Δ		7532	6338	4374	5203	1791

* Macrophages incubated with HSV-Ag; i.e. pulsing as described in the Materials and Methods section.

† 5×10^4 T cells prepared as described in the Materials and Methods section.

‡ Mean c.p.m. ± s.e.

§ Incremental c.p.m.

HLA-D/DR restriction of the T cell response to HSV-Ag

To study this question, different allogeneic mixtures of HLA-D/DR identical, semi-compatible and incompatible mixtures of T cells and macrophages were used. The results of two typical experiments are shown in Table 2. Both T cell donors had clinical evidence of recurrent herpes labialis and demonstrated a macrophage-dependent T cell response to HSV-Ag using Ag pulsed macrophages. It can be seen that when mixing their T cells with allogeneic macrophages, the sharing of both HLA-D/DR determinants between the responding T cell and macrophage donors reveals a response close to that seen in the autologous combination. The sharing of only one HLA-D/DR determinant between the macrophage and the T cell donor results in a moderate to strong HSV response. However, where there is total HLA-D/DR disparity between the two donors, only a slight antigen-specific response is seen in both experiments.

To study whether there were any differences between direct addition of HSV-Ag to the T/Mø mixture and pulsing of the macrophages with antigen, we performed parallel experiments using the same T cell donor. The results of one typical experiment out of three can be seen in Table 3. The top part (I) of the Table gives the results after pulsing of macrophages while the bottom part (II) reveals the results after direct addition of HSV-Ag to the macrophage/T cell mixtures. Both approaches demonstrate essentially the same findings as described in Table 2; i.e. the allogeneic antigen presenting capability of the macrophages decreases with the decreasing number of HLA-D/DR determinants shared between the T cell and macrophage donor. It appears that the sharing of both HLA-D/DR antigens with the T cell donor gives a higher RAgS using the pulsed approach. However, this was not a constant finding throughout the other experiments.

In Fig. 1 the results of another experiment are illustrated. In this, soluble antigen has been added directly to the T-Mø cell mixtures. Again it is demonstrated that when both HLA-D/DR antigens are shared between the responding T cell and the macrophage donors a strong HSV-Ag-specific response occurs. In cases where only one HLA-D/DR antigen is shared between the two donors, a more variable response is seen. In cases of total HLA-D/DR disparity, however, the response is poor.

TABLE 3. HLA-D restriction of T cell response to HSV-Ag pulsing of macrophages with HSV-Ag (expt I) compared to non-pulsing (expt II)*

	HSV-Ag	T cells† alone	T cells+Mø‡ autologous	T cells+Mø allogenic			
			DR 2/5	DR 2/5	DR 2/-	DR 5/8	DR 1/8
I	+	70±23§	5778±436	5128±410	2977±352	3294±243	1668±171
	−	174±36	326±53	399±119	816±27	1115±202	2472±279
	Δ¶		5452	4729	1272	2139	
	RAgS** (%)		100	87	23	39	
II	+	70±23	7527±1726	4672±611	4120±387	3915±910	6503±753
	−	174±36	98±16	149±38	502±179	592±77	5574±1756
	Δ		7429	4523	3618	3323	929
	RAgS (%)		100	61	49	45	13

* Pulsing (expt I) and non-pulsing (expt II) with HSV-Ag as described in the Materials and Methods section.

† 5×10^4 T cells prepared as described in the Materials and Methods section.

‡ Pulsing of macrophages with HSV-Ag as described in the Materials and Methods section. For the non-pulsed approaching $2{\cdot}5\times10^3$ macrophages prepared as described in the Materials and Methods section have been used.

§ Mean c.p.m.±s.e.

¶ Incremental c.p.m.

** Relative antigen-specific stimulation.

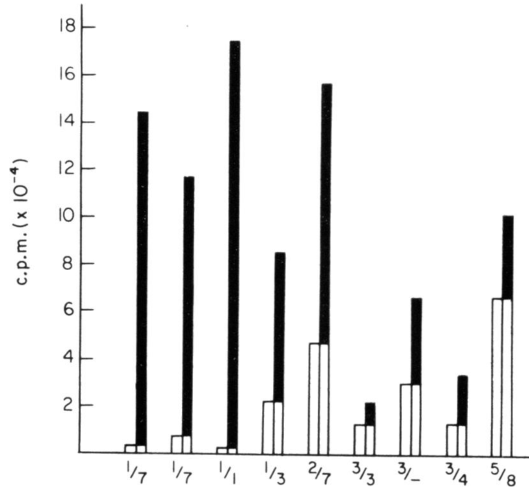

FIG. 1. HLA-D restriction of the HSV response. Along the abscissa are shown the HLA-D/DR types of the autologous (e.g. 1/7) and allogeneic macrophage donors. Response with HSV-Ag (■) expressed as median of triplicates. (□) Response without antigen.

Fig. 2 summarizes our results of the allogeneic mixing experiments. The sharing of both HLA-D/DR determinants between the T cell and macrophage donors reveals a median relative antigen stimulation (RAgS) of about 90% of that seen in autologous combinations whereas a sharing of one determinant yields approx. 45% and sharing of none, approx. 20%.

Lack of co-operation is not due to suppression or generation of cytotoxic T cells

In our experiments we found a lack of co-operation between T cells and allogeneic HLA-D/DR disparate macrophages. It may be argued that this could be due to suppression of the T cell response due to the allogeneic MLC response caused by the HLA-D disparity. Another possibility would be killing of the HLA-D/DR disparate macrophages by generation of cytotoxic T cells in the allogeneic mixtures.

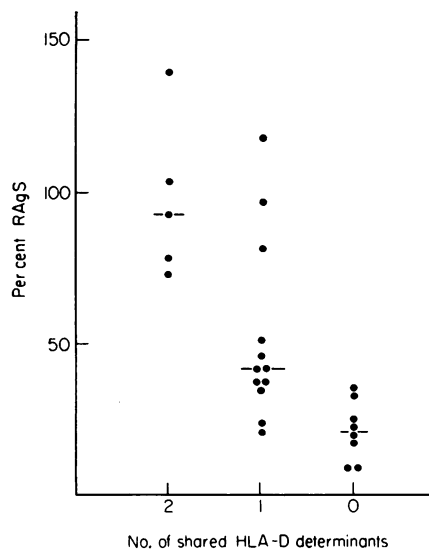

FIG. 2. Combined results. Relative antigen stimulation is expressed as per cent RAgS, the autologous being set at 100%. The number of shared HLA-D/DR determinants between T cell and macrophage donor is shown in the abscissa.

TABLE 4. Autologous T/Mø response to HSV-Ag. Influence of allogeneic macrophages

T cell*	Mø†	Herpes virus antigen‡ +	Herpes virus antigen‡ −	Increment§
A	A	31,713	662	31,051
A	B	23,083	17,264	5819
A	A+B	45,013	12,796	33,217
C	C	19,764	568	19,196
C	D	21,735	17,460	4275
C	C+D	31,732	12,241	19,491

A and B are fully HLA-D incompatible, C and D are fully HLA-D incompatible.

* 5×10^4 T cells prepared as described in the Materials and Methods section.

† 5×10^3 macrophages prepared as described in the Materials and Methods section.

‡ Final HSV-Ag concentration 1:100.

§ Incremental c.p.m. expressed as median of triplicates.

To exclude any of these possibilities the following experiments were carried out. First, HSV-Ag-responding T cells were co-cultured with autologous as well as HLA-D/DR disparate allogeneic macrophages, alone or in mixture. The results of two such experiments out of four are seen in Table 4. The T cell donors A and C both react with a macrophage-dependent T cell response when stimulated with HSV-Ag. The addition of HLA-D/DR incompatible macrophages alone results in a strong mixed lymphocyte reaction due to HLA-D/DR disparity, and these macrophages are poorly able to induce an HSV-Ag-specific response. The important point, however, is that these allogeneic incompatible macro-

TABLE 5. Pre-culture of macrophages alone or with HLA-D/DR disparate T cells: capability of macrophages to present HSV-Ag to autologous T cells compared to macrophages pre-cultured alone

T cell* donor	HSV-Ag†	Autologous macrophages‡ −	Autologous macrophages‡ +	Autologous macrophages after 6 days of pre-incubation: Alone	Autologous macrophages after 6 days of pre-incubation: With third party T cells
A	−	322	437	141	6111
	+	349	43,231	17,898	18,123
	Δ§	27	42,794	17,767	12,012
C	−	188	770	135	1575
	+	349	6347	4421	5037
	Δ	161	5577	4287	3575

* 5×10^4 T cells prepared as described in the Materials and Methods section.

† Final HSV-Ag concentration 1:100.

‡ 5×10^3 macrophages prepared as described in the Materials and Methods section.

§ Incremental c.p.m.

phages do not suppress the HSV-Ag-specific T cell response in the presence of autologous macrophages. The right hand column of the table, demonstrates that the incremental c.p.m. is approximately the same whether allogeneic macrophages are present or not in the autologous combination of T and Mø.

To investigate whether formation of cytotoxic T cells might explain our results, we carried out experiments where the macrophages from the HSV-sensitive T cell donor were first pre-cultured alone or with T cells from HLA-D/DR disparate donors for 6 days. After this period the cell mixtures were irradiated and freshly prepared autologous T cells from the macrophage donor and HSV-Ag in a final concentration of 1:100 were added. The results of two such experiments are depicted in Table 5. It can be seen that the pre-culture of macrophages together with HLA-D/DR disparate T cells did not result in any significantly decreased capability of the macrophages to present HSV-Ag to the autologous T cells compared to macrophages pre-cultured alone.

DISCUSSION

Our results demonstrate that the macrophage-dependent T cell response to HSV-Ag *in vitro* is restricted by self-HLA-D/DR molecules of the T cell donor. An HSV-Ag-specific T cell response will occur only when HSV-Ag is presented to the T cells from sensitized donors together with adherent Ag-presenting cells sharing both or at least one of the HLA-D/DR determinants with the T cell donor. The source of macrophages in these cases, however, is of less importance. Macrophages from HSV-sensitized as well as from non-sensitized individuals may serve as antigen-presenting cells for the responding T cells providing these allogeneic macrophages share HLA-D/DR determinants with the T cell donor.

Whether soluble antigen be present in the T-Mø cell mixture or the macrophages be pulsed apparently is of minor importance. The HSV-Ag-specific T cell response reveals comparable results using either approaches. In both instances the responding T cells probably recognize HLA-D and the foreign HSV-Ag in the membrane of the antigen-presenting cell. In some of our experiments we did, however, find a lower stimulation expressed as crude incremental c.p.m. when pulsed macrophages were used. This is most likely explained by loss of antigen and antigen presenting cells through the washing procedure.

We have shown previously that a strong T cell response to HSV-Ag requires only a small number of antigen-presenting cells, giving responses even at T:Mø ratio of 50:1 (Berle & Thorsby, 1979). We took advantage of this in some of the studies with allogeneic macrophages. In cases where the MLR response between HLA-D/DR disparate cell donors was marked, the HLA-D/DR restricted HSV-specific T cell response was sometimes masked. If in these cases the macrophage concentration was lowered from the usual 1:10 to 1:20, the HLA-D/DR-restricted T cell response was demasked, due to decreased background MLR. Although this ratio is below the one existing *in vivo*, even this small number of macrophages is capable of triggering the HSV-Ag-specific T cell response.

In co-culturing T cells with allogeneic HLA-D/DR disparate macrophages, we find only slight or no response, reflecting incapability of these allogeneic macrophages to function as Ag-presenting cells. It may well be argued that this lack of co-operation reflects suppression or cytotoxicity rather than being caused by self HLA-D/DR restriction. Theoretically, either suppressing factors may be formed during the allogeneic interaction or the HLA-D/DR disparate macrophages might induce cytotoxic T cells killing the allogeneic macrophages and thus removing most of the necessary adherent cells. In the first case, the additional presence of fully HLA-D/DR disparate allogeneic macrophages in the autologous T+Mø mixture should suppress the virus-specific T cell response. This does not, however, appear to be the case. Our results show that there is no difference between the T cell response whether third party allogeneic macrophages are present or not.

The second possibility also is made highly unlikely by our experiments since pre-culturing the macrophages from a given T cell donor together with allogeneic HLA-D/DR disparate T cells did not significantly reduce the ability of these macrophages to induce an HSV-specific response when autologous T cells and antigen were added to the cell culture. The obtained results are comparable to the experiments where macrophages had been pre-cultured alone for the same period of time.

In other experiments we have shown that in HLA-D/DR heterozygous individuals, two different antigen-specific T cell clones exist, the one with specificity for antigen together with the HLA-D/DR determinant inherited from the one parent, and another clone with the same antigen specificity, but restricted to the other inherited HLA-D/DR determinant (Hirschberg, Bergh & Thorsby, 1979). By inspection of Fig. 2, one can see that in general, in combinations where the T cell and macrophage donors share only one of the HLA-D/DR determinants, a response close to 50% of that seen in autologous or in combinations sharing both HLA-D/DR determinants was seen. This would be in accordance with the above mentioned finding of separate HLA-D/DR restricted antigen-specific clones in HLA-D/DR heterozygous individuals.

Taken together, our results demonstrate that the proliferative response of T cells from sensitized donors to HSV-Ag is restricted by self HLA-D/DR molecules. The results are very similar to those which we have found previously for the proliferative T cell response to PPD (Bergholtz & Thorsby 1977, 1978). However, the experiments using HSV-Ag generally resulted in a higher antigen-specific response and less background stimulation of the T cells without adherent cells added. Studies with both antigens demonstrate, that to obtain a secondary response *in vitro*, the T cells must recognize antigen together with one or both of the HLA-D/DR molecules which were present during initial sensitization *in vivo*. Based on studies in the mouse (Zinkernagel, 1978), this HLA-D/DR restriction is in all probability imposed on the T cells during intrathymic development and thus prior to encounter with antigen.

Having found HLA-D/DR restriction of the proliferative response against HSV-Ag, it would be of interest to study whether cytotoxicity be restricted also by HLA antigens, possibly the HLA-ABC antigens. This will be the subject of further studies.

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