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The effects of changes at peptide residues contacting MHC class II T-cell receptor on antigen recognition and human Th0 cell effector function

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SUMMARY

Cytokines can influence the selection of functional subsets (Th1 or Th2) of $CD4^+$ T cells. However, quantitative changes in affinity of peptide/major histocompatibility complex (MHC) class II/T-cell receptor (TCR) interactions may alter antigen density and modulate T-cell effector function. The possibility exists to use peptide analogues to induce a partial signal to dissociate production of interleukin-4 (IL-4) and interferon-γ (IFN-γ) by T-helper type-0 (Th0) cells and, consequently, to regulate T-cell function. Based on binding assays and resolution of the crystalline structure of an influenza virus haemagglutinin peptide (HA 306–318) bound to the human MHC class II molecule DRB1*0101, we synthesized HA peptide analogues with amino acid substitutions predicted to modify either MHC class II/peptide density or TCR/peptide interactions. When we examined their antigenicity using cloned human Th0 cells, the analogues, in general, elicited a gradation in potency reflected by a reduction in both proliferation and cytokine production (IL-2, IL-4 and IFN-γ). Although the analogue HA-R^{309} diminished IL-2 production, none of the analogues tested could selectively induce only IL-4 or IFN-γ. Since, in general, the effector functions of the Th0 cells examined here were resistant to selective manipulation by the peptide analogues, this suggests that for some clones of chronically activated T cells modulation of selected functions may be difficult to achieve.

INTRODUCTION

The activation of $CD4^+$ T cells follows engagement of T-cell antigen receptor (TCR) by complexes of peptide fragments of antigen associated with major histocompatibility complex (MHC) class II molecules,[1,2] combined with co-stimulatory signals mediated by the interaction of accessory molecules.[3,4] Polarity in the functional activity of the $CD4^+$ T-cell subsets, termed Th1 and Th2, is demonstrated by their ability to promote cell-mediated immunity or humoral immunity, respectively.[5] The functional penotype of these T-cell subsets is characterized by their cytokine profiles: Th1 cells secrete interferon-γ (IFN-γ) and interleukin-2 (IL-2), whereas Th2 cells secrete IL-4, IL-5 and IL-6. An intermediate subset, Th0 cells, which secretes both Th1 and Th2 cytokines may constitute the major functional component of the T-cell repertoire in humans.[6] Depending on which subset is predominantly activated may determine whether the immune response to antigen is protective or pathogenic. For example, exposure of atopic individuals to environmental allergens induces Th2 cells and the formation of allergen-specific IgE, while in non-atopic subjects, T-cell responses of a Th1 phenotype generate no pathological changes.[7]

Mechanisms that determine the selection of effector function of $CD4^+$ T cells are only partially defined. The presence of selected cytokines in the local microenvironment[8,9] and the antigen-presenting cell (APC) type[10,11] may influence differentiation into distinct subsets. It has also been reported that antigen density and the affinity of peptide/MHC interactions with the TCR affect the balance of Th1 and Th2 cells.[12–14] The specificity of MHC class II molecules may contribute to the induction of either the Th1 or Th2 pathways. For example, after immunization with human collagen type IV, mice expressing $I\text{-}A^s$ generate Th1 responses, while in $I\text{-}A^b$ mice Th2 responses dominate.[13,14] The functional phenotype of mature T cells may be selectively manipulated by stimulation with peptide analogues that bind with similar affinity to MHC class II molecules but have altered affinities for TCR. These analogues of immunogenic peptides termed altered peptide ligands (APL), have amino acid substitutions at critical TCR contact residues, such that they may have agonist, partial

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Abbreviations: APC, antigen-presenting cell; APL, altered peptide ligand; c.p.m., counts per minute; HA, haemagglutinin; PBMC, peripheral blood mononuclear cells; [^{3}H]TdR, tritiated methyl thymidine; TCR, T-cell receptor; Th, T-helper cell.

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agonist or antagonist effects on T-cell activation.[15] In one study, when partial agonists were used to stimulate mouse T-cell clones, effector function and the proliferative response were uncoupled.[16] Thus, there is evidence to suggest that altering the affinities of immunogenic peptides may modify T-cell effector function.

Based on these findings, we were interested in the possibility of using peptide analogues that induce a partial signal to uncouple the production of IL-4 and IFN-γ in functionally differentiated Th0 cells and, therefore, manipulate their effector function. We have previously reported a similar phenomenon in human $CD4^+$ T cells in which cytokine production was selectively regulated following stimulation with high concentrations of peptide.[17] Clearly, these observations have a potentially important therapeutic role in the redirection of aberrant immune responses, such as those induced by the exposure of atopic individuals to allergens.

The analysis of the interaction of an influenza virus haemagglutinin peptide (HA residues 306–318) and its analogues with HLA-DR1 class II molecules in binding assays,[18,19] together with the recent resolution of the crystal structure of HA 306–318 occupying the combining site of DRB1*0101,[20] offered an opportunity to test the effects of peptide analogues on the regulation of T-cell function. By synthesizing peptide analogues of HA 306–318 we have determined the influence of altered affinities for MHC class II (DR1) and/or TCR on antigen recognition and the selective induction of IL-4 or IFN-γ secretion by cloned human Th0 cells.

MATERIALS AND METHODS

Antigens

The influenza virus haemagglutinin (HA) peptide residues 306–318 (PKYVKQNTLKLAT) and analogues (Table 1) were synthesized with an Applied Biosystems 431A synthesizer by standard solid phase methods, as described previously.[21]

*Isolation of HA 306–318-reactive DRB1*0101-restricted cloned human T cells*

Cloned T cells of HA1.7 were isolated by limiting dilution cloning as described previously.[22] The T-cell clone was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco, Life Technologies, Paisley, UK) and 5% inactivated human A^+ serum (National Blood Transfusion Service, Edgware, UK) by stimulating with HA 306–318 peptide and irradiated histocompatible peripheral blood mononuclear cells (PBMC; 5×10^5/ml; 2500 rads) as a source of APC, together with IL-2 (10% v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). In all experiments, clones were tested 7–8 days after the last addition of antigen and APC.

Proliferation assays

Cloned T cells (HA1.7; $2{\cdot}0 \times 10^4$/well) were cultured in round-bottomed 96-well microtitre trays (Nunc, Gibco Life Technologies) with analogues of HA 306–318 (0·003–30 μg/ml) in the presence of mitomycin c-treated murine fibroblasts expressing HLA-DRB1*0101 (2×10^4 cells/well). Recombinant IFN-γ or IL-4 (gifts from DNAX Research Institute, Palo Alto, CA) was added for the duration of the cultures at concentrations of 50 U/ml and 40 ng/ml, respectively. The cultures were pulsed with tritiated thymidine (1 μCi/well; [^{3}H]TdR; Amersham International Plc., Amersham, UK) after 48 hr and harvested 8–16 hr later. Proliferation was determined by [^{3}H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as mean c.p.m. for triplicate cultures with SEM < 25%.

Measurement of IFN-γ, IL-4 and IL-2

In parallel with the proliferation assays, plates were prepared in duplicate and supernatants were collected following 24 hr and 72 hr incubation. The triplicate wells were pooled and IFN-γ and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA; Eurogenetics, Teddington, UK), and IL-2 levels were determined in biological assays with CTLL murine cell line. Briefly, supernatants were incubated with CTLL (5×10^3/well) in triplicate for 24 hr. Proliferation was determined by pulsing the wells for 6 hr with 1 μCi [^{3}H]TdR and incorporation measured by liquid scintillation spectroscopy. The level of IL-2 as calibrated against a standard curve of recombinant IL-2. Each experiment was performed on at least three separate occasions and results are presented from single representative experiments.

RESULTS

Effect of amino acid substitutions in HA 306–318 on proliferation and cytokine production by cloned Th0 cells

The interaction of the native peptide and analogues of HA

Table 1. Amino acid sequence of wild-type peptide and analogues of HA 306–318

306		308	309			312						318
P	K	Y	V	K	Q	N	T	L	K	L	A	T
–	–	I	–	–	–	–	–	–	–	–	–	–
–	–	V	–	–	–	–	–	–	–	–	–	–
–	–	S	–	–	–	–	–	–	–	–	–	–
–	–	–	Q	–	–	–	–	–	–	–	–	–
–	–	–	R	–	–	–	–	–	–	–	–	–
–	–	–	E	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	F	–	–	–	–	–	–
–	–	–	–	–	–	K	–	–	–	–	–	–
–	–	–	–	–	–	E	–	–	–	–	–	–

306–318 with affinity-purified and membrane-expressed HLA class II molecules of different specificity, including DRB1*0101, has been investigated elsewhere.[18,19] Furthermore, crystallographic analysis of HA 306–318 occupying the antigen-binding site of the human class II molecule DRB1*0101 has been reported.[20] From the information accumulated collectively from these different experimental approaches, it has been suggested that the tyrosine at position 308 interacts with the MHC molecules and is accommodated in a hydrophobic pocket, the specificity of which is determined, in part, by the glycine at position 86 in the DR1 β-chain.[18] Position 309 (valine) is predicted to contact surface residues of DRB1*0101 but is exposed and may, therefore, also interact with the TCR. Position 312 (asparagine) appears to point away from the MHC and also potentially interacts with the TCR.

Substitutions at position 308

Stimulation with the natural HA peptide over a concentration range in the presence of mouse fibroblasts expressing DRB1*0101 induced maximum proliferation of the HA-specific T cells at 3 μg/ml, whereas at higher doses of peptide (30 μg/ml) T-cell proliferation was reduced (Fig. 1a). A conservative substitution of tyrosine (Y^{308}) by isoleucine (I^{308}) induced a similar dose–response curve (Fig. 1a).

It is predicted[18–20] that amino acid substitutions at position 308 may alter the binding characteristics of the peptide to DRB1*0101 depending upon the properties of the residue introduced. With changes in binding affinity the slope of the dose–response curves would remain unchanged but the concentration of peptide required to induce proliferation relative to the natural peptide would differ. However, the proliferative responses to HA-V^{308} did not follow this pattern, and the slope of the curve was altered (Fig. 1a). HA-V^{308} was less antigenic than HA-Y^{308} at 0·003 μg/ml of peptide, giving c.p.m. (%SEM) of 5520 (15) and 11420 (3), respectively. At higher concentrations (e.g. 3 μg/ml), proliferation was enhanced with c.p.m. of 20675 (7) for HA-Y^{308} and 33289 (10) for HA-V^{308}. At these higher concentrations the formation of complexes between HA-V^{308} and DRB1*0101 that were more stable than those formed with the natural peptide may contribute to the pattern of proliferation we observed. As previously reported,[18,19,23] HA-S^{308} has substantially reduced binding to DRB1*0101 class II molecules and is less antigenic than HA-Y^{308} (Fig. 1a).

The ability of the HA analogues (Y^{308}, I^{308}, S^{308} and V^{308}) to modulate IL-2, IL-4 and IFN-γ production was also investigated. The levels of IL-2 present in 24 hr culture supernatants paralleled the proliferative curves, with the exception of HA-V^{308} which induced enhanced proliferation

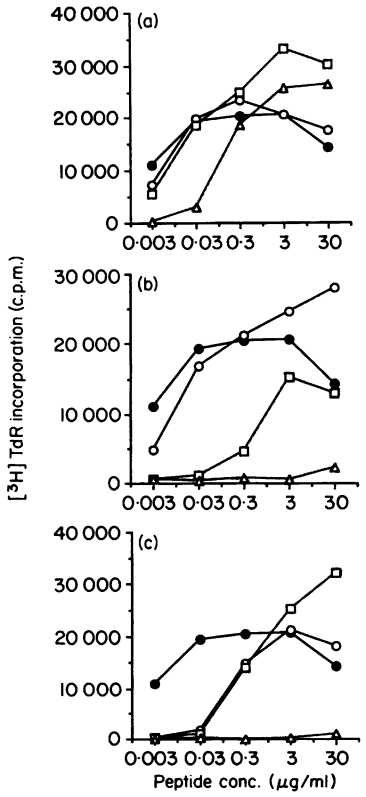

Figure 1. Antigenicity of the HA 306–318 analogues. Cloned T cells were stimulated with peptides in the presence of mitomycin c-treated murine HLA-DRB1*0101$^+$ murine fibroblasts. [^{3}H]TdR incorporation was determined at 72 hr. (a) Native peptide (●), I^{308} (○), V^{308} (□), S^{308} (△); (b) native peptide (●), Q^{309} (○), R^{309} (□), E^{309} (△); (c) native peptide (●), S^{312} (○), F^{312} (□), K^{312} (△).

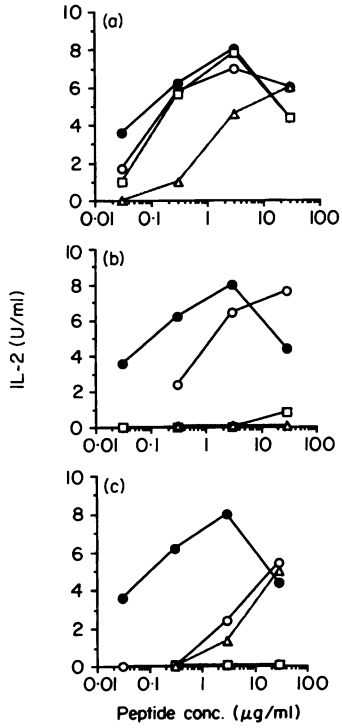

Figure 2. Effect of HA peptide analogues on IL-2 production. Supernatants were collected at 24 hr from cloned T cells stimulated with peptide analogues in the presence of DRB1*0101$^+$ murine fibroblasts. Levels of IL-2 were determined in bioassays using CTLL cells. (a) Native peptide (●), I^{308} (○), V^{308} (□), S^{308} (△); (b) native peptide (●), Q^{309} (○), R^{309} (□), E^{309} (△); (c) native peptide (●), F^{312} (○), K^{312} (□), S^{312} (△).

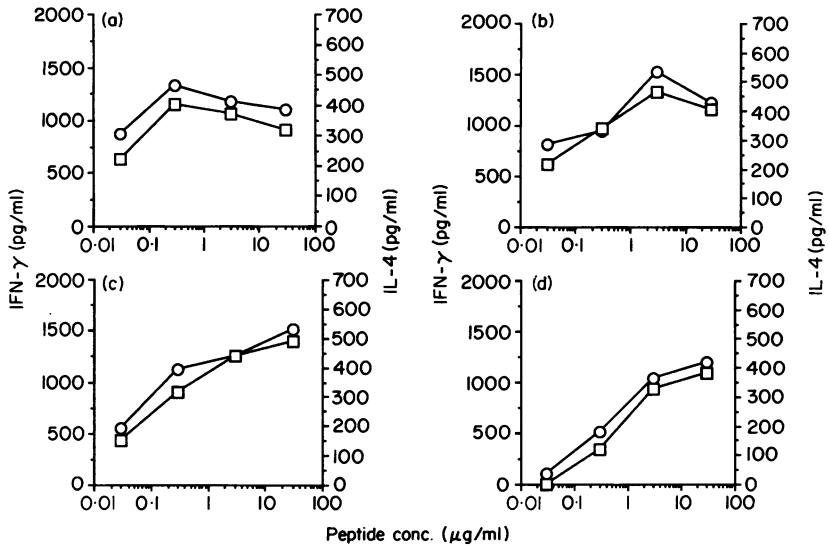

Figure 3. Effect of HA peptide analogues with substitutions at 308 on IL-4 and IFN-γ production. (a) Native peptide, (b) I^{308}, (c) V^{308}, (d) S^{308}. IFN-γ (O) and IL-4 (□) levels were assayed by ELISA.

but not IL-2 release compared to the natural peptide (Fig. 2a). Similarly, the analogues that induced different levels of proliferation induced similar dose–response curves for the production of IFN-γ and IL-4 and the kinetics of secretion remained unaltered (Fig. 3). Analysis of the kinetics suggested that maximal IL-4 was produced at 24 hr. In contrast, IFN-γ was secreted throughout the culture period (data not shown). This suggested that cytokine synthesis was closely linked to the strength of the signal mediated via the TCR, and that altering the affinity of the interaction of the HA peptide with DRB1*0101 did not differentially regulate IFN-γ, IL-2 or IL-4 production. There were differences in overall cytokine production by the HA analogues, as illustrated by the dose–response curves relating to the antigenicity of the individual analogues. The dose–response curves observed in the proliferation and cytokine assays did not necessarily have the same slope but, in general, high levels of proliferation were reflected by high levels of cytokine production. However, at 72 hr for the HA-I^{308} and HA-V^{308} the levels of IL-4 were lower at high concentrations of peptide but IFN-γ production plateaued (data not shown).

Substitution at position 309

It is suggested that HA-V^{309} may interact with both MHC class II and TCR molecules.[18–20] Replacing valine with glutamine (Q) at position 309 altered the slope of the dose–response curve and at the highest concentration HA-Q^{309} was markedly more stimulatory than the natural peptide, 14351 (19) c.p.m. (%SEM) compared to 28008 (6) c.p.m. for HA-Q^{309} (Fig. 1b). In contrast, the proliferation induced by HA-R^{309} was diminished and only at 30 μg/ml was the response equivalent to the native peptide. When glutamic acid (E) was present at position 309 no stimulation was observed. As with the HA-308 analogues, IL-2 production by the 309-substituted analogues at 24 hr was, in general, proportional to proliferation and reflected antigenicity. However, detectable IL-2 was not produced by cells stimulated with HA-R^{309}, even at doses that induced marked proliferation (Fig. 2b).

The HA peptide analogues with substitutions at position 309 had similar effects on IFN-γ and IL-4 production to those observed with the HA-308 analogues (Fig. 4). In general, those peptides that induced strong proliferation (HA-Q^{309}) also induced high levels of cytokines, whereas less antigenic peptides (HA-R^{309}) resulted in the production of lower levels of both IFN-γ and IL-4. HA-E^{309}, which failed to stimulate proliferation, was also unable to trigger cytokine secretion. A log–linear relationship between antigen concentration and cytokine production was observed for HA-R^{309} and HA-Q^{309}, with the latter being more potent.

Substitutions at position 312

Asparagine (N) at position 312 would appear to point away from the combining site and, therefore, potentially interacts with the TCR and not with the HLA class II molecule. HA-S^{312} was at least two logs less efficient at inducing proliferation and the antigen dose–response curve was shifted to the right compared to that of native peptide (Fig. 1c). At low antigen concentrations HA-F^{312} was less stimulatory, whereas with higher doses proliferation was enhanced compared to that induced by the native peptide. Proliferation for HA-F^{312} at 30 μg/ml was 32160 (8) c.p.m. (%SEM) compared to 14351 (19) for native HA 306–318. HA-K^{312} failed to stimulate proliferation.

Levels of IL-2 reflected the antigenicity of the analogues, as determined by proliferation (Fig. 2c). The effects upon production of IFN-γ and IL-4 with HA-312 analogues were similar to those observed with analogues with substitutions at positions 308 and 309 (Fig. 5). The levels of the cytokines produced were proportional to the strength of stimulation and there was no differential secretion of either IFN-γ or IL-4 in response to alterations in the residue at position 312. HA-F^{312},

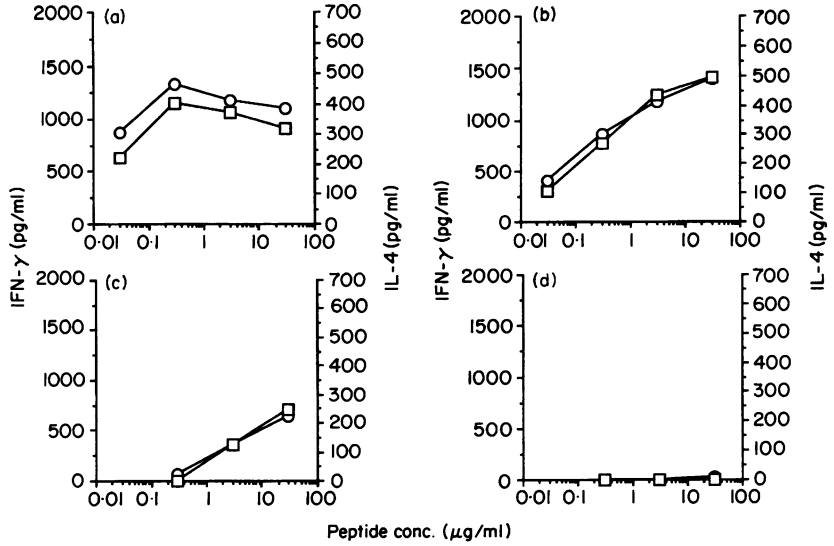

Figure 4. Effect of HA peptide analogues with substitutions at 309 on IL-4 (□) and IFN-γ (○) production. (a) Native peptide, (b) Q^{309}, (c) R^{309}, (d) E^{309}.

which induced maximal proliferation, triggered the highest amounts of cytokines.

Effects of co-culture with IFN-γ or IL-4 on the production of these cytokines by Th0 cells stimulated with the HA analogues

The addition of IFN-γ or IL-4 to the cultures failed to modulate the proliferative response of HA-specific T cells to the natural peptide or the HA analogues (data not shown). IL-4 levels were measured at 24 and 72 hr in those assays in which T cells were cultured with IFN-γ, APC and selected peptide analogues. Conversely, in cultures where T cells were stimulated with the peptide analogues together with IL-4, IFN-γ production was determined. Co-culture with IL-4 had a minimal effect upon the levels of IFN-γ at 24 hr but, in general, they were reduced at 72 hr (Fig. 6). One exception was that co-culture of the T cells with IL-4 and HA-Q^{309} resulted in a small increase in the levels of IFN-γ. Stimulation with the analogues and IFN-γ reduced the levels of IL-4 induced in all cases (Fig. 7). That the addition of exogenous IFN-γ or IL-4 failed to dissociate the production of either cytokine by the HA-specific T cells probably reflects the fact that this is a fully differentiated T-cell clone.

DISCUSSION

In the experiments reported here analogues of HA 306–318 with different affinities for DRB1*0101 or TCR were examined

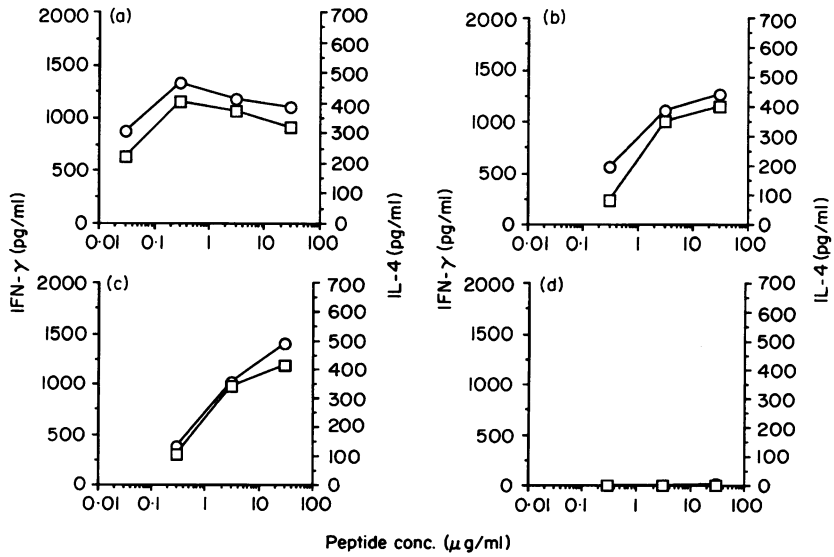

Figure 5. Effect of HA peptide analogues with substitutions at 312 on IL-4 (□) and IFN-γ (○) production. (a) Native peptide, (b) S^{312}, (c) F^{312}, (d) K^{312}.

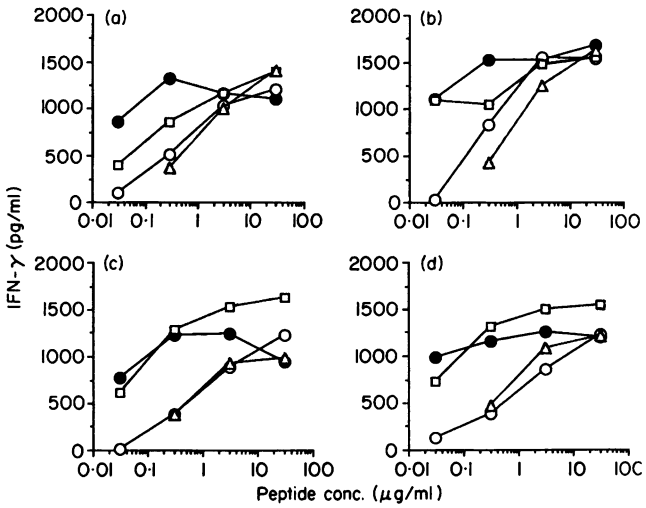

Figure 6. Kinetics of IFN-γ production induced by HA peptide analogues alone and in the presence of IL-4. IFN-γ production was determined at 24 (a) and 72 (b) hr for cloned T cells stimulated with APC and peptide analogues: native peptide (●), S^{308} (○), Q^{309} (□), F^{312} (△). IL-4 (40 ng/ml) was added for the duration of the cultures and IFN-γ measured at 24 (c) and 72 hr (d).

for altered antigenicity and their ability to regulate proliferation and cytokine production independently for a clone of HA-specific human Th0 cells. The results of HLA class II binding studies[18,19,21] and the resolution of the crystal structure of HLA-DRA/B1*0101 with HA 306–318[20] prompted us to construct peptide analogues with amino acid substitutions in positions predicted to form important interactions with MHC class II molecules, TCR or both. Pockets were identified in the groove of DR1 that were capable of binding five residues of the peptide.[20] The critical and largest pocket is formed and regulated by the polymorphic residue 86 in the β-chain.

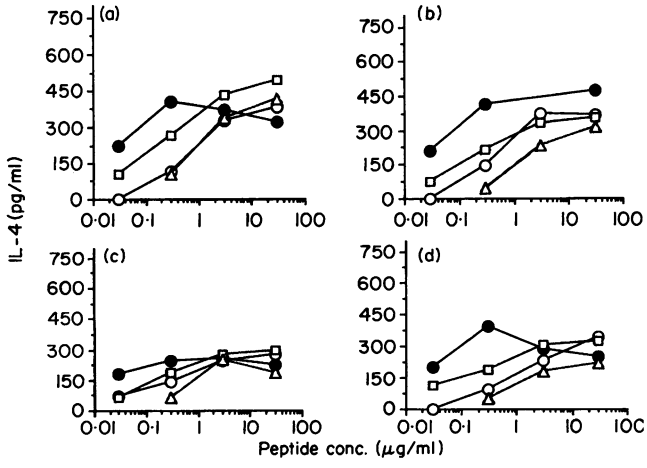

Figure 7. Kinetics of IL-4 production induced by HA peptide analogues alone and in the presence of IFN-γ. IL-4 production was determined at 24 (a) and 72 (b) hr for cloned T cells stimulated with APC and peptide analogues: native peptide (●), S^{308} (○), Q^{309} (□), F^{312} (△). IFN-γ (50 U/ml) was added for the duration of the cultures and IL-4 measured at 24 (c) and 72 hr (d).

DRB1*0101 carries a glycine in this position, selecting for large hydrophobic residues such as the tyrosine in position 308 of the HA peptide. This confirmed earlier observations of the importance of this residue for binding to DR1[18,19,23,24] and was the initial choice for substitution in order to determine the effect of altering the affinity of peptide binding.

The side chains of residues P^{306}, Y^{308}, V^{309}, K^{310}, K^{315} and A^{317} were also predicted to make contact with HLA-DR1, but were significantly exposed to the solvent allowing possible contact with TCR. The alkyl side chain of V^{309} protrudes at an angle horizontal to the peptide backbone, with one methyl group contacting residues of the α helix of the β-chain of DR1. The other methyl group is angled up towards the solvent and possibly contacts the TCR, thus substitution of this position in the peptide allowed for a more subtle modulation of the peptides to bind to DR1 with an addition effect on TCR recognition. However, by altering DR1 binding residues, allosteric effects may modify the molecular surface required for TCR recognition. In order to compensate, substitutions of N^{312}, whose side chain is directed vertically and makes no contact with the groove, should solely alter the affinity of the TCR binding.

Previous studies using alanine substitutions in the natural peptide sequence of 11 of the 13 residues of HA 306–318 were not able to eliminate HLA-DR binding.[24] Boehncke *et al.*[25] concluded that epitope recognition resulting in T-cell activation required interactions between only a small number of residues. This prompted us to determine if modification of HA-306–318 at positions 308, 309 and 312 has critical effects on T-cell recognition and effector function.

The cloned T cells (HA1.7) had a Th0 cytokine response profile and the gradation in antigenicity of HA analogues was reflected, in general, by a reduction in proliferation accompanied by decreased IL-2, IL-4 and IFN-γ production. Importantly, in response to altered peptide antigenicity no differences were observed in the release of IL-4 and IFN-γ, which predominantly determine the selective functional activities of Th1 and Th2 cells. T-cell proliferation was reduced in the presence of high concentrations of the natural peptide and the majority of HA analogues, with a dose dependence that paralleled their antigenicity. Selected substitutions ($Y \rightarrow V^{308}$, $V \rightarrow Q^{309}$ and $N \rightarrow F^{312}$) resulted in a gain in functional activity, suggesting the removal of a negative effect of the natural residue, rather than introducing an amino acid that enhances the affinity of the peptide for MHC class II or TCR.[25] Alternatively, the complexes formed between these analogues and DRB1*0101 may be more stable due to a conformational change. Modified T-cell recognition of ovalbumin peptide analogues that were substituted at positions buried in the MHC class I peptide binding groove was suggested to be the result of an alteration in the conformation of the complex.[26] One effect of conformational modification may be to change on/off rates and/or the stability of MHC/TCR/peptide interactions.

High affinity interactions of the ligand with TCR results in efficient signalling through CD3, while low affinity interactions may result in binding but impaired signalling. From our results it appears that the effector functions of the mature Th0 cells examined in this study could not be readily uncoupled by changes in ligand/receptor affinities or in antigen density. The effects on effector function of those analogues in which MHC

class II or TCR contact residues had been substituted paralleled their antigenicity. However, we observed that HA-R^{309}, although less antigenic than the wild-type sequence in terms of stimulating proliferation, induced the release of IL-4 and IFN-γ but not IL-2. Furthermore, the addition of IL-4 reduced the capacity of the analogues to induce IFN-γ, except for HA-Q^{309} when IFN-γ production was minimally enhanced at 24 hr. Thus, signals mediated by cytokines also make a major contribution to T-cell activation. Studies on murine CD4$^+$ T-cell clones have demonstrated that different dose thresholds are required for distinct effector functions.[27] If the effector functions of these T cells require different thresholds this may help to explain the uncoupling of effector function achieved by stimulation with APL of different affinities and antigenicity.[16,27] However, in general, we have not been able to demonstrate altered effector function due to different signalling thresholds of a human Th0 clone. Alternatively, the ability to modulate function may be characteristic only of T cells with polarized effector function, such as Th2 and Th1 and not Th0 cells. It has been proposed that quantitative differences in the threshold requirements for IL-2 and IL-3 production are related to the more potent synergy of costimulation necessary for the induction of IL-2. The affinity of the ligand/receptor interactions may affect recruitment of accessory molecules and susceptibility to costimulatory activity. It is possible that the costimulatory signals required for the activation of Th0 cells, at least for this particular T-cell clone, are less important.

Our results imply that different effector functions of some human Th0 T cells are resistant to selective manipulation by peptide analogues with substitutions that alter interactions between peptide, MHC class II and TCR. This suggests that switching the functional phenotype of T cells by stimulation with peptide analogues may be difficult to achieve for certain T-cell subsets, which has implications for the design of peptides for immunotherapy.

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