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# Primary response of naive CD4$^+$ T cells to amino acid-substituted analogs of an antigenic peptide can show distinct activation patterns: Th1- and Th2-type cytokine secretion, and helper activity for antibody production without apparent cytokine secretion

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**Abstract Naive CD4$^+$ T cells differentiate into two types of helper T cells showing an interferon-γ-predominant (Th1) or an interleukin-4-predominant (Th2) cytokine secretion profile after repeated antigenic stimulation. Their differentiation can be influenced by slight differences in the interaction between the T cell receptor (TCR) and its ligand at the time of primary activation. However, the primary response of freshly isolated naive CD4$^+$ T cells to altered TCR ligands is still unclear. Here, we investigated the primary response of splenic naive CD4$^+$ T cells derived from transgenic mice expressing TCR specific for residues 323–339 of ovalbumin (OVA323–339) bound to I-A$^d$ molecules. Naive CD4$^+$ T cells secreted either Th1- or Th2-type cytokines immediately after stimulation with OVA323–339 or its single amino acid-substituted analogs. Helper activity for antibody secretion by co-cultured resting B cells was also found in the primary response, accompanied by either low-level Th2-type cytokine secretion or no apparent cytokine secretion. Our results clearly indicate that dichotomy of the Th1/Th2 cytokine secretion profile can be elicited upon primary activation of naive CD4$^+$ T cells. We also demonstrate that the helper activity of naive CD4$^+$ T cells for antibody production does not correspond to the amounts of the relevant cytokines secreted.**



*Key words:* Altered peptide ligand; Naive CD4$^+$ T cell; Primary response; Th1/Th2; Antibody production; Cytokine production

## 1. Introduction

CD4$^+$ T cells recognize antigenic peptides bound to major histocompatibility complex (MHC) class II molecules through their T cell receptor (TCR). Naive CD4$^+$ T cells activated upon stimulation with specific antigens start to differentiate into two distinct types of effector cells each showing a particular cytokine secretion profile and particular immune regulatory functions [1]. Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ, and lymphotoxin and contribute to cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, and IL-10, and are efficient in providing help for antibody production by B cells.

A large number of studies have revealed that T cell responses are modified upon stimulation of these cells with certain amino acid-substituted analogs of an antigenic peptide, as these substitutions result in slight changes in the interaction between TCR and the peptide/MHC complexes. These peptides are called altered peptide ligands [2]. Recent studies have shown that the altered TCR/peptide/MHC interaction strongly affects the differentiation of naive CD4$^+$ T cells into Th1 or Th2 cells. Bottomly et al. [3] have demonstrated that naive CD4$^+$ T cells cultured in the presence of a wild-type peptide or one of its single amino acid-substituted analogs during primary culture secreted Th1- or Th2-type cytokines, respectively, after secondary stimulation with the wild-type peptide. It has also been demonstrated that varying the dose of antigen used in primary stimulation affects the Th1/Th2 differentiation of naive CD4$^+$ T cells [4,5]. These results suggest that differences in the primary response of naive CD4$^+$ T cells influence their differentiation into Th1/Th2 cells. However, the previous studies exclusively focused on the secondary cytokine response of naive CD4$^+$ T cells, and thus it is unclear whether freshly isolated naive CD4$^+$ T cells secrete Th1- or Th2-type cytokines after recognizing specific antigens for the first time in vitro. It is also unknown whether naive CD4$^+$ T cells exhibit other activation characteristics besides cytokine secretion.

In this study, we used naive CD4$^+$ T cells from OVA23-3 transgenic mice [6] which express TCR α and β chains specific for a peptide corresponding to residues 323–339 of ovalbumin (OVA323–339) in complex with an I-A$^d$ molecule, a murine MHC class II molecule. We investigated the primary activation of naive CD4$^+$ T cells in response to a panel of 40 single amino acid-substituted analogs of OVA323–339. These analog peptides each carried a substituted amino acid residue at a putative TCR contact site. Here, we demonstrate that naive CD4$^+$ T cells can exhibit a Th1- or Th2-type cytokine response upon primary activation, depending on the amino acid sequence of the antigenic peptide. We also found that

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*Abbreviations:* APC, antigen-presenting cell; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; OVA, ovalbumin; TCR, T cell receptor


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naive CD4$^+$ T cells can be activated to elicit antibody production without significant cytokine secretion.

## 2. Materials and methods

### 2.1. Animals

OVA323–339-specific TCR transgenic mice (OVA23-3) with the BALB/c genetic background were produced as described previously [6] and maintained by backcrossing to BALB/c mice in our animal facility. BALB/c mice (female, 6 weeks old) were purchased from Japan CLEA Inc. (Tokyo, Japan).

### 2.2. Synthetic peptides

OVA323–339 was prepared by solid-phase peptide synthesis using an automated peptide synthesizer model 430A (PE Applied Biosystems, Foster City, CA, USA), and purified by reverse-phase HPLC. A series of single amino acid-substituted analogs of OVA323–339 (Table 1) was purchased from Chiron Mimotopes Pty Ltd (Clayton, Vic., Australia).

### 2.3. Preparation of naive CD4$^+$ T cells, resting B cells, and antigen-presenting cells

Splenic CD4$^+$ T cells were isolated from OVA23-3 mice by positive selection using anti-CD4-FITC monoclonal antibodies (GK1.5, PharMingen, San Diego, CA, USA) and a MACS anti-FITC multisort kit (Miltenyi Biotech, Bergisch Gladbach, Germany). To obtain naive CD4$^+$ T cells, the CD4$^+$ T cells were further separated into cells expressing high levels of CD62L (a naive T cell marker) by positive selection using anti-CD62L microbeads (Miltenyi Biotech). Splenic B cells were purified from BALB/c mice by positive selection using anti-B220 microbeads (Miltenyi Biotech). The B cells were further fractionated into small resting B cells (60/70% interface) on discontinuous Percoll gradients (50/60/70%). T cell-depleted antigen-presenting cells (APCs) were prepared from spleen cells of BALB/c mice by negative selection using anti-Thy1.2 microbeads (Miltenyi Biotech), and were treated with 50 μg/ml mitomycin C (Sigma, St. Louis, MO, USA). Isolated naive CD4$^+$ T cells, resting B cells, and APCs were routinely $>96\%$ CD4$^+$CD62L$^{high}$, $>98\%$ B220$^+$, and $<5\%$ Thy1.2$^+$, respectively.

### 2.4. T cell proliferation assay

Naive CD4$^+$ T cells ($1\times10^5$/well) in a total volume of 200 μl were stimulated with each peptide at 5 μM in the presence of APCs ($3\times10^5$/well) in 96-well flat bottom plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). The cells were cultured for 96 h. Proliferation was assessed by measuring the incorporation of [$^3$H]thymidine (1 μCi/well) added for the final 24 h of culture.

### 2.5. Cytokine secretion

Naive CD4$^+$ T cells ($1\times10^5$/well) in a total volume of 200 μl were stimulated with each peptide at 5 μM in the presence of APCs

Table 1
List of peptides used in this study

| Peptide designation | Amino acid sequence[a] | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 323 | | | | | | | 330 | | | | | | | | | 339 |
| OVA323–339[b] | Ile | Ser | Gln | Ala | Val | His | Ala | Ala | His | Ala | Glu | Ile | Asn | Glu | Ala | Gly | Arg |
| A326S | – | – | – | Ser | – | – | – | – | – | – | – | – | – | – | – | – | – |
| A326L | – | – | – | Leu | – | – | – | – | – | – | – | – | – | – | – | – | – |
| A326Q | – | – | – | Gln | – | – | – | – | – | – | – | – | – | – | – | – | – |
| A326Y | – | – | – | Tyr | – | – | – | – | – | – | – | – | – | – | – | – | – |
| A326E | – | – | – | Glu | – | – | – | – | – | – | – | – | – | – | – | – | – |
| A326R | – | – | – | Arg | – | – | – | – | – | – | – | – | – | – | – | – | – |
| H328K | – | – | – | – | – | Lys | – | – | – | – | – | – | – | – | – | – | – |
| H328Y | – | – | – | – | – | Tyr | – | – | – | – | – | – | – | – | – | – | – |
| H328Q | – | – | – | – | – | Gln | – | – | – | – | – | – | – | – | – | – | – |
| H328L | – | – | – | – | – | Leu | – | – | – | – | – | – | – | – | – | – | – |
| H328E | – | – | – | – | – | Glu | – | – | – | – | – | – | – | – | – | – | – |
| H328S | – | – | – | – | – | Ser | – | – | – | – | – | – | – | – | – | – | – |
| A329S | – | – | – | – | – | – | Ser | – | – | – | – | – | – | – | – | – | – |
| A329L | – | – | – | – | – | – | Leu | – | – | – | – | – | – | – | – | – | – |
| A329Q | – | – | – | – | – | – | Gln | – | – | – | – | – | – | – | – | – | – |
| A329Y | – | – | – | – | – | – | Tyr | – | – | – | – | – | – | – | – | – | – |
| A329E | – | – | – | – | – | – | Glu | – | – | – | – | – | – | – | – | – | – |
| A329R | – | – | – | – | – | – | Arg | – | – | – | – | – | – | – | – | – | – |
| A330S | – | – | – | – | – | – | – | Ser | – | – | – | – | – | – | – | – | – |
| A330L | – | – | – | – | – | – | – | Leu | – | – | – | – | – | – | – | – | – |
| A330Q | – | – | – | – | – | – | – | Gln | – | – | – | – | – | – | – | – | – |
| A330Y | – | – | – | – | – | – | – | Tyr | – | – | – | – | – | – | – | – | – |
| A330E | – | – | – | – | – | – | – | Glu | – | – | – | – | – | – | – | – | – |
| A330R | – | – | – | – | – | – | – | Arg | – | – | – | – | – | – | – | – | – |
| H331K | – | – | – | – | – | – | – | – | Lys | – | – | – | – | – | – | – | – |
| H331Q | – | – | – | – | – | – | – | – | Gln | – | – | – | – | – | – | – | – |
| H331L | – | – | – | – | – | – | – | – | Leu | – | – | – | – | – | – | – | – |
| H331E | – | – | – | – | – | – | – | – | Glu | – | – | – | – | – | – | – | – |
| E333D | – | – | – | – | – | – | – | – | – | – | Asp | – | – | – | – | – | – |
| E333Q | – | – | – | – | – | – | – | – | – | – | Gln | – | – | – | – | – | – |
| E333L | – | – | – | – | – | – | – | – | – | – | Leu | – | – | – | – | – | – |
| E333A | – | – | – | – | – | – | – | – | – | – | Ala | – | – | – | – | – | – |
| E333Y | – | – | – | – | – | – | – | – | – | – | Tyr | – | – | – | – | – | – |
| E333R | – | – | – | – | – | – | – | – | – | – | Arg | – | – | – | – | – | – |
| I334L | – | – | – | – | – | – | – | – | – | – | – | Leu | – | – | – | – | – |
| I334Q | – | – | – | – | – | – | – | – | – | – | – | Gln | – | – | – | – | – |
| I334S | – | – | – | – | – | – | – | – | – | – | – | Ser | – | – | – | – | – |
| I334Y | – | – | – | – | – | – | – | – | – | – | – | Tyr | – | – | – | – | – |
| I334D | – | – | – | – | – | – | – | – | – | – | – | Asp | – | – | – | – | – |
| I334R | – | – | – | – | – | – | – | – | – | – | – | Arg | – | – | – | – | – |

[a]Dashes indicate identity with the OVA323–339 sequence.
[b]A peptide corresponding to region 323–339 of OVA.

($3\times10^5$/well) in 96-well flat bottom plates. The culture supernatants were recovered after 24 h for measurement of IL-2 or after 72 h for IL-4 and IFN-γ. The cytokine content was determined by means of a two-site enzyme-linked immunosorbent assay (ELISA) as described previously [7]. The pairs of primary and biotinylated secondary capture antibodies used were as follows: JES6-1A12 (PharMingen) and JES6-5H4 (PharMingen) for IL-2, BVD4-1D11 (PharMingen) and BVD6-24G2 (PharMingen) for IL-4, and R4-6A2 and XMG1.2 [7] for IFN-γ.

*2.6. Antibody production*

Naive $CD4^+$ T cells ($1\times10^5$/well) and resting B cells ($1\times10^5$/well) in a total volume of 200 μl were co-cultured with each peptide at 5 μM in the presence of APCs ($3\times10^5$/well) in 96-well flat bottom plates. The culture supernatants were recovered after 7 days. The levels of total IgM and IgG were measured by two-site ELISA as described previously [8] except that goat anti-IgM antibody (Cappel-Organon Teknika, Durham, NC, USA) and alkaline phosphatase-conjugated goat anti-IgM antibody (Zymed, South San Francisco, CA, USA) were used as the primary and secondary capture antibodies, respectively.

## 3. Results

A study examining the crystal structure of the OVA323–339/I-$A^d$ molecule complex has revealed that the side groups of residues $^{327}$Val and $^{332}$Ala are critical for I-$A^d$ binding and that those of other residues in the region 325–334 seem to interact with TCR [9]. On the basis of this information, we prepared analog peptides with a single amino acid substitution at one of seven putative TCR contact residues, $^{326}$Ala, $^{328}$His, $^{329}$Ala, $^{330}$Ala, $^{331}$His, $^{333}$Glu, and $^{334}$Ile (Table 1).

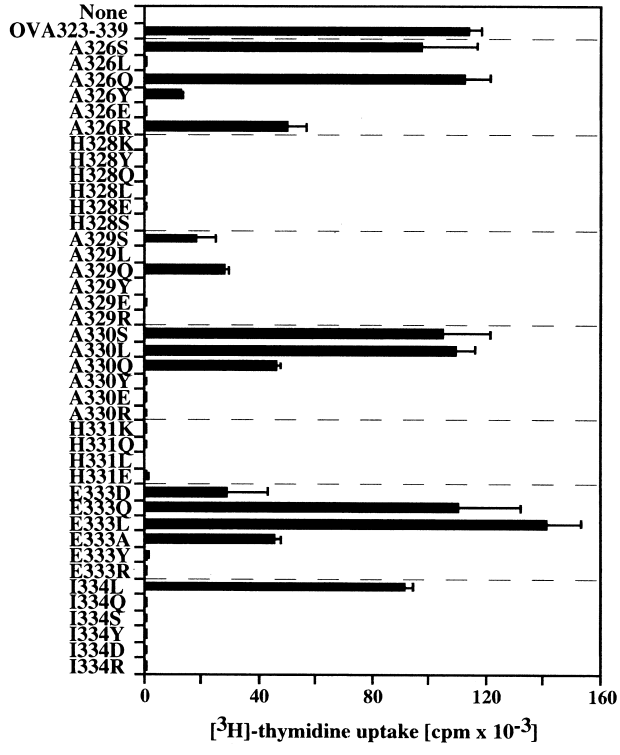


Fig. 1. Proliferation of naive $CD4^+$ T cells in response to single amino acid-substituted analogs of OVA323–339. Naive $CD4^+$ T cells ($1\times10^5$/well) were stimulated with each peptide at 5 μM in the presence of APC ($3\times10^5$/well) for 96 h, and [$^3$H]thymidine uptake during the last 24 h was measured. The results shown are representative of three independent experiments. The value (cpm ± S.D.) of the background indicated as None was 267 ± 4.

We investigated the primary activation profile of naive $CD4^+$ T cells upon stimulation with each peptide by measuring proliferation (Fig. 1), cytokine secretion (Fig. 2), and the ability to provide help for antibody secretion by B cells (Fig. 3). All assays were performed at a single peptide concentration to exclude the dose effect. A relatively high concentration, 5 μM, was selected, since our preliminary experiments showed that some of the peptides that elicited a T cell response at 5 μM induced no detectable response at lower concentrations. At this peptide concentration (5 μM), OVA323–339 induced strong proliferation, and efficient secretion of IL-2 and IFN-γ, which is a typical Th1-type response. The wild-type peptide did not activate T cells to provide help to resting B cells to secrete antibodies.

No analog peptide tested with a substitution at residue $^{328}$His or $^{331}$His induced proliferation, cytokine secretion, or antibody secretion. Likewise, analogs with a substitution at residue $^{334}$Ile elicited no T cell response, except for I334L, having a highly conservative substitution, which induced these responses to the same extent as OVA323–339. These results show that residues $^{328}$His, $^{331}$His, and $^{334}$Ile are absolutely required for recognition by the TCR in our system.

In contrast, some analogs with a substitution at residue $^{326}$Ala, $^{329}$Ala, $^{330}$Ala, or $^{333}$Glu induced T cell responses qualitatively different from those induced by OVA323–339. A326Q induced strong proliferation and IL-4 secretion without production of IL-2 and IFN-γ, which is a typical Th2-type response. A326R also elicited the Th2-type response, but more weakly than A326Q. A326R activated T cells to provide help to resting B cells to secrete large amounts of IgM and IgG, whereas A326Q induced lower levels of IgM and IgG secretion. A326S having a more conservative substitution induced the same response as that induced by OVA323–339. A326Y elicited very weak proliferation and a weak antibody response with no apparent cytokine production. A326L and A326E elicited no T cell response.

Among the analogs with a substitution at residue $^{329}$Ala, only A329S and A329Q elicited weak T cell responses. A329S induced a cytokine response similar to the typical Th2-type response elicited by A326R except for a low level of IL-2 secretion. This peptide also elicited a very weak antibody response, as compared with that elicited by A326R. A329Q elicited weak proliferation and antibody secretion without cytokine secretion.

With regard to the analogs with a substitution at residue $^{330}$Ala, A330Q elicited responses quite similar to those elicited by A329S. A330S and A330L having a conservative substitution induced the same responses as those induced by OVA323–339, whereas A330Y, A330E, and A330R elicited no T cell response.

Finally, the analogs with a substitution at residue $^{333}$Glu induced various types of responses. E333A induced proliferation and cytokine responses very similar to those observed in the case of A326R but a weaker IgG response. The response induced by E333D was quite distinct in that there was no detectable cytokine secretion and only weak proliferation, but an unexpectedly high level of antibody secretion. E333L elicited strong proliferation and a weak antibody response, with low-level secretion of IL-2 and IFN-γ, and modest IL-4 secretion. E333Q elicited the same responses as those observed in the case of OVA323–339. E333Y and E333R elicited no T cell response.

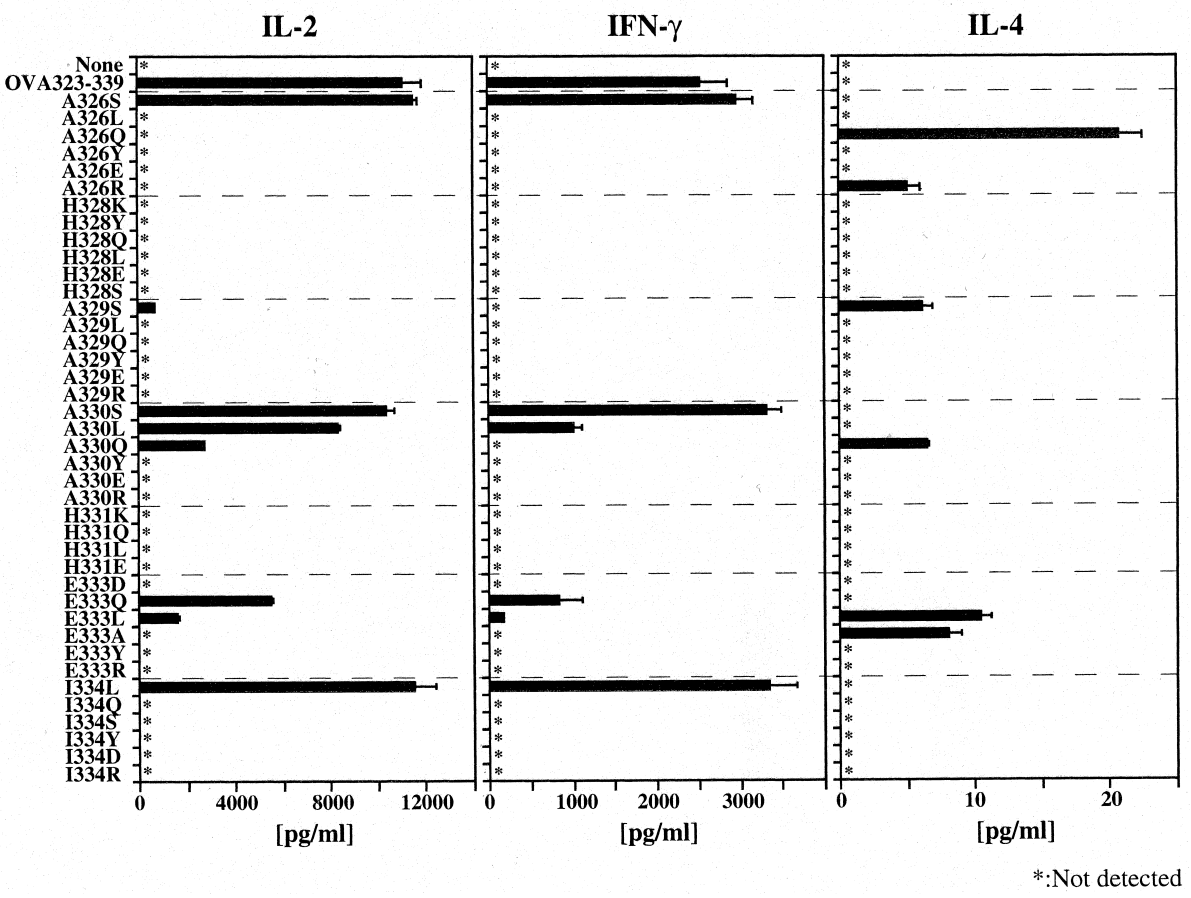


Fig. 2. Cytokine secretion by naive CD4$^+$ T cells upon primary stimulation with analog peptides. Naive CD4$^+$ T cells ($1\times10^5$/well) were stimulated with each peptide at 5 μM in the presence of APC ($3\times10^5$/well). The culture supernatants were recovered after 24 h or 72 h for measurement of IL-2 or IL-4 and IFN-γ, respectively. The cytokine levels were determined by ELISA. The results shown are representative of three independent experiments. The detection limit was 313 pg/ml for IL-2, 93.7 pg/ml for IFN-γ, and 1.9 pg/ml for IL-4.

## 4. Discussion

In the present study, we assessed the primary response of naive CD4$^+$ T cells upon stimulation with analog peptides having a single amino acid substitution. The primary response of naive CD4$^+$ T cells elicited by altered TCR ligands (Figs. 1–3) can be classified into at least four types in addition to a null response (Table 2): (A) a Th1-type cytokine response with strong proliferation and no induction of antibody production, (B) a Th2-type cytokine response with strong proliferation and inefficient induction of antibody production, (C) a weak Th2-type cytokine response with weak proliferation and efficient induction of antibody production, and (D) no detectable cytokine response but with weak proliferation and efficient induction of antibody production. These results clearly show that naive CD4$^+$ T cells can display a Th1- or Th2-type cytokine secretion profile immediately after primary stimulation with altered peptide ligands. We also demonstrate that naive CD4$^+$ T cells can be activated to elicit antibody production, even if they do not secrete detectable levels of cytokines. Our study has revealed for the first time the distinct activation profiles of naive CD4$^+$ T cells displayed in their primary response upon stimulation with altered TCR ligands.

Previous studies demonstrating differentiation of naive CD4$^+$ T cells into Th1/Th2 subsets in vitro did not show such distinct cytokine responses upon primary stimulation of freshly isolated naive T cells [10,11]. In those studies, the cytokine levels following secondary stimulation were about 200–500-fold higher than those observed after primary stimulation in the present study. Very low levels of cytokine secretion in the primary response such as we observed may have been ignored or undetectable in those previous studies. Otherwise, the levels of cytokine secretion in the primary response may vary depending on the strain of TCR-transgenic mice and/or the individual mice used in each experimental system. Nevertheless, it is obvious from our results that low levels but distinct Th1- or Th2-type cytokine secretion profiles can be induced in the primary response of naive CD4$^+$ T cells, and these are considered to drive naive CD4$^+$ T cells to differentiate into effector Th1/Th2 cells in an autocrine manner.

Our analysis of T cell responses to analog peptides have revealed that the residues $^{328}$His, $^{331}$His, and $^{334}$Ile of OVA323–339 are essential for the interaction with transgenic TCR in OVA23-3 mice. It was previously shown that $^{331}$His is commonly required for recognition by the TCR of several OVA323–339-specific T cells [12]. Furthermore, analysis of the crystal structure of the OVA323–339/I-A$^d$ complex [9] has shown that $^{328}$His and $^{331}$His are located in positions adequate for TCR binding. On the other hand, substitution at residue $^{326}$Ala, $^{329}$Ala, $^{330}$Ala, or $^{333}$Glu led to several changes in the responses of naive CD4$^+$ T cells, although substitution at residue $^{329}$Ala or $^{330}$Ala was less effective in

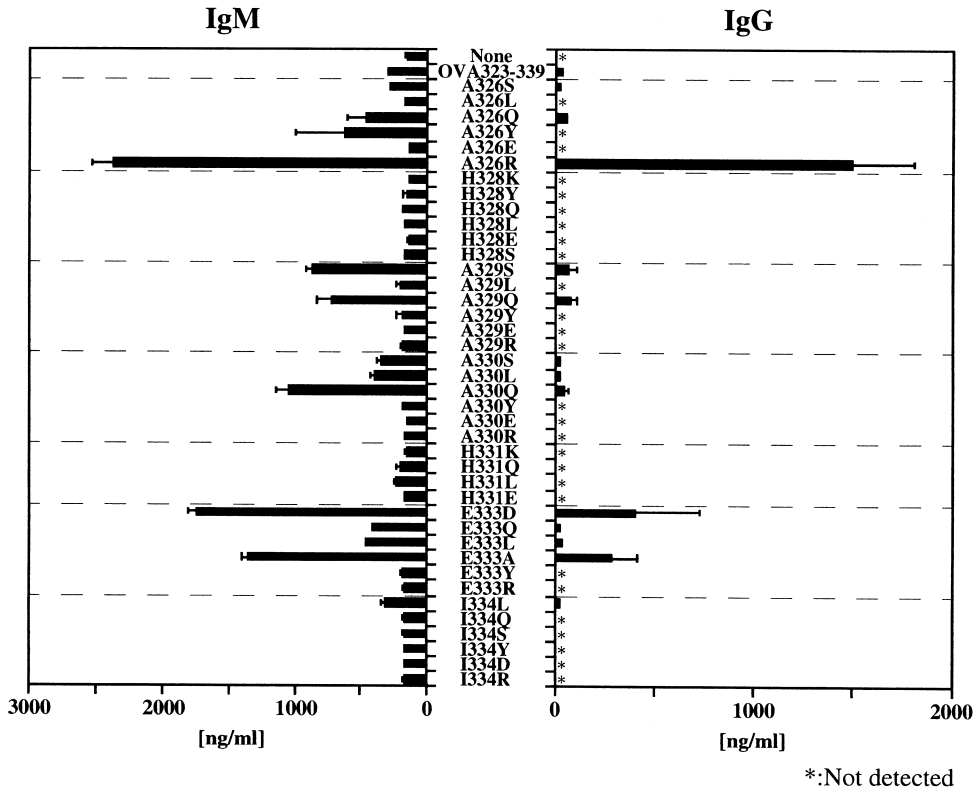


Fig. 3. Antibody production by B cells co-cultured with naive CD4$^+$ T cells upon stimulation with analog peptides. Naive CD4$^+$ T cells ($1\times10^5$/well) and resting B cells ($1\times10^5$/well) were stimulated with each peptide at 5 μM in the presence of APC ($3\times10^5$/well). The culture supernatants were recovered after 7 days. The levels of total IgM and IgG were measured by ELISA. The results shown are representative of three independent experiments. The detection limit was 39 ng/ml for IgM and 15 ng/ml for IgG.

causing these changes. Allen et al. [13] have found a hierarchy in the importance of individual amino acid residues contacting TCR in an antigenic peptide. The primary contact residues are indispensable for binding to TCR, and the secondary contact sites are defined as TCR contact sites which are assumed to play a lesser role in the interaction and are permissive for substitutions. When we adapt our findings to this hypothetical hierarchy, $^{328}$His, $^{331}$His, and $^{334}$Ile seem to be the primary contact residues, and $^{326}$Ala, $^{329}$Ala, $^{330}$Ala, and $^{333}$Glu seem to be the secondary contact residues in our system. When some amino acid substitution was introduced at these secondary TCR contact residues, T cell responses could be modified in terms of the cytokine response pattern and/or antibody production helping activity.

Changes in the T cell response patterns were highly dependent on the side groups of the amino acid residues substituted for the wild-type ones. We focused on substitutions at residues $^{326}$Ala, $^{329}$Ala, $^{330}$Ala and $^{333}$Glu where some substitutions caused a qualitative change in the T cell response. All analog peptides with a substitution of a charged residue (Glu or Arg) for Ala at residue 326, 329 or 330 completely lost the capability to activate T cells, except for A326R which elicited a type C response as described in Table 2. Similarly, four out of six analogs with a substitution of an amino acid having a hydrophobic side chain with a large molecular mass (Leu or Tyr) for an Ala residue induced no T cell response. Therefore, substitution of an amino acid residue having a large molecular mass or a charged side chain for Ala seems to have a tendency to abolish the capability to elicit a T cell response. In contrast, all peptides with a substitution of a non-charged polar amino acid (Ser or Gln) for Ala retained the capability to elicit some T cell response. Four out of six analogs with such a substitu-

Table 2
Summary of the types of primary responses displayed by naive CD4$^+$ T cells

| Type of response | Representative peptide[a] | Proliferation | Cytokine secretion | | | Helper activity for antibody secretion |
|---|---|---|---|---|---|---|
| | | | IL-2 | IFN-γ | IL-4 | |
| A | OVA323–339 | +++[b] | +++ | +++ | − | − |
| B | A326Q | +++ | − | − | +++ | + |
| C | A326R | ++ | − | − | + | +++ |
| D | E333D | + | − | − | − | ++ |

[a]Other peptides which elicited each type of response are as follows: (A) A326S, A330S, A330L, E333Q, and I334L; (B) E333L; (C) A329S, A330Q, and E333A; and (D) A326Y and A329Q. The rest of the peptides tested elicited no response.
[b]+++, ++, + or −, means $>70\%$, 40–70%, 10–40%, or $<10\%$, respectively, of the maximal response induced by the peptide with the highest efficiency in inducing each response.

tion induced a qualitative change in the T cell response (A326Q, type B; A329S and A330Q, type C; A329Q, type D). As for analog peptides with a substitution at residue $^{333}$Glu, half of them caused a qualitative change in the T cell response (E333L, type B; E333A, type C; E333D, type D). Taken together, it appears that modification at the position of a charged residue or substitution of a non-charged polar amino acid residue for a small non-polar amino acid residue in an antigenic peptide would be a good approach to modify T cell responses elicited by the peptide qualitatively. Since we did not test all possible substitutions at the relevant residues, a general rule regarding the relationship between amino acid substitution and alteration of T cell responses could not be established. However, when the 3D structure of the complex of OVA323–339/I-A$^d$ and the TCR expressed in our transgenic mice has been revealed, the present findings will be useful to elucidate the structural basis for the modified T cell response induced by altered peptide ligands.

An unexpected finding in this study was that an antibody response can be elicited even if the T cells secrete no detectable cytokines. To produce antibodies in response to T cell-dependent antigens, B cells require two signals derived from activated T cells [14,15]. The first one is mediated by cytokines, especially those secreted from Th2 cells, such as IL-4. The second signal is mediated by interaction between cell surface molecules on T cells and B cells. Molecules expressed on activated T cells, such as CD40 ligand, OX40 and Fas ligand, are important for regulation of T cell-dependent activation of B cells [16,17]. Our results show that the extent of IL-4 production by T cells stimulated with the analog peptides was not simply correlated with their antibody production-inducing activity, although it is still possible that an undetectable level of the cytokine was secreted upon stimulation with the antibody-eliciting peptides. Thus it is likely that the patterns of expression of each of the surface molecules rather than the Th2-type cytokine response of T cells may be modulated by analog peptides through modification of the TCR/antigen/MHC interaction, resulting in different antibody responses of B cells. Further analysis of the levels of expression of these molecules will help to clarify the precise mechanism which controls the different antibody responses of B cells induced by altered peptide ligands. In our present experiments, we investigated the antibody response of polyclonal B cells interacting with T cells specific for OVA323–339, as a primary step in the study. We should further examine the antigen-specific interactions between T cells and B cells with the same antigen specificity. We expect that studies using analog peptides eliciting each of the four types of responses illustrated in Table 2 will shed light on the precise mechanism underlying T cell helper activity for the antibody response.

## References

[1] Mosmann, T.R. and Coffman, R.L. (1989) Annu. Rev. Immunol. 7, 145–173.
[2] Sloan-Lancaster, J. and Allen, P.M. (1996) Annu. Rev. Immunol. 14, 1–27.
[3] Tao, X., Grant, C., Constant, S. and Bottomly, K. (1997) J. Immunol. 158, 4237–4244.
[4] Hosken, N.A., Shibuya, K., Heath, A.W., Murphy, K.M. and O'Garra, A. (1995) J. Exp. Med. 182, 1579–1584.
[5] Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. and Bottomly, K. (1995) J. Exp. Med. 182, 1591–1596.
[6] Sato, T., Sasahara, T., Nakamura, Y., Osaki, T., Hasegawa, T., Tadakuma, T., Arata, Y., Kumagai, Y., Katsuki, M. and Habu, S. (1994) Eur. J. Immunol. 24, 1512–1516.
[7] Hisatsune, T., Nishijima, K., Minai, Y., Kohyama, M. and Kaminogawa, S. (1994) Cell. Immunol. 154, 181–192.
[8] Sakurai, T., Ametani, A., Nakamura, Y., Shimizu, M., Idota, T. and Kaminogawa, S. (1993) Int. Immunol. 5, 793–800.
[9] Scott, C.A., Peterson, P.A., Teyton, L. and Wilson, I.A. (1998) Immunity 8, 319–329.
[10] Boutin, Y., Leitenberg, D., Tao, X. and Bottomly, K. (1997) J. Immunol. 159, 5802–5809.
[11] Leitenberg, D., Boutin, Y., Constant, S. and Bottomly, K. (1998) J. Immunol. 161, 1194–1203.
[12] Sette, A., Buus, S., Colon, S., Smith, J.A., Miles, C. and Grey, H.M. (1987) Nature 328, 395–399.
[13] Evavold, B.D., Sloan-Lancaster, J. and Allen, P.M. (1993) Immunol. Today 14, 602–609.
[14] Parker, D.C. (1993) Annu. Rev. Immunol. 11, 331–360.
[15] Clark, E.A. and Ledbetter, J.A. (1994) Nature 367, 425–428.
[16] Cleary, A.M., Fortune, S.M., Yellin, M.J., Chess, L. and Lederman, S. (1995) J. Immunol. 155, 3329–3337.
[17] Stuber, E., Neurath, M., Calderhead, D., Fell, H.P. and Strober, W. (1995) Immunity 2, 507–521.