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Immunology

Differential induction of interferon γ gene expression after activation of CD4+ T cells by conventional antigen and Mls superantigen

(T helper subsets/cytokine gene expression/Mls recognition)

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ABSTRACT **We have analyzed cytokine gene expression by a murine CD4+ T-cell clone that expresses three forms of T-cell recognition. The clone employs a V_β6-containing T-cell receptor to recognize (*i*) a self class II major histocompatibility complex and an ovalbumin-derived peptide (OVA), (*ii*) an I-A^b alloantigen, and (*iii*) *Mls-1*^{a}. All three responses are accompanied by similar levels of cell proliferation. However, although interferon γ gene expression is strongly induced during both physiological recognition of the OVA peptide and allogeneic major histocompatibility complex recognition, expression of this gene was not detected during the Mls response. These studies indicate that Mls recognition is functionally distinct from T-cell recognition of peptides and alloantigens and leads to an alternative pattern of cytokine gene expression. They also suggest the possibility that encounter with these two classes of T-cell antigen *in vivo* may generate subsets of T helper cells that display different patterns of cytokine gene expression.**

T-cell recognition can be divided into two major types. Conventional forms of recognition include the interaction between the T-cell receptor (TCR) and an antigenic peptide embedded in the individual's major histocompatibility complex (MHC) product (1, 2), termed physiological recognition, as well as the interaction between the TCR and polymorphic variants of MHC molecules, termed allorecognition. Although the generation of alloreactive clones is not well understood, there is evidence that T-cell clones initially selected for receptors that bind to antigenic peptides and self MHC products use these same receptors to recognize cross-reactive epitopes on allogeneic MHC products (3, 4).

A second type of T-cell recognition comes from studies of the Mls^a locus. An extremely high frequency of CD4+ T-cell clones respond to B-cell-specific Mls determinants in association with many different polymorphic variants of class II MHC products (5–7). The absence of typical MHC restriction and the apparently independent segregation of receptors allowing Mls recognition from receptors for conventional antigen (8) are two surprising features of this type of T-cell response. The biological consequences of Mls recognition are also distinct from both allogeneic recognition and physiological recognition of minor H antigens. For example, Mls responses do not normally result in homograft rejection or graft vs. host reactions (9). These and other unusual features of the Mls response have led to suggestions that this form of T-cell recognition may be qualitatively distinct from both physiologic and allogeneic recognition (8, 10) and more similar in nature to recognition of bacterial superantigens (11, 12).

In this report, we have analyzed cytokine gene expression after activation of a murine CD4+ T-cell clone termed O3 that expresses all three forms of T-cell reactivity (13, 14). The clone employs a V_β6-containing TCR to recognize (*i*) self class II MHC products (I-A^d) and an ovalbumin-derived peptide (OVA), (*ii*) an I-A^b alloantigen, and (*iii*) *Mls-1*^{a} determinants in association with several class II MHC gene products. All three responses elicit similar levels of cell division. However, measurement of cytokine gene expression showed that although interferon γ (IFN-γ) is strongly induced after both physiological and allogeneic MHC recognition, expression of this gene does not accompany the Mls response.

EXPERIMENTAL PROCEDURES

Animals and Monoclonal Antibodies. BALB/c, C57BL/6J, and DBA/2J mice were obtained from The Jackson Laboratory and maintained at the Redstone Animal Facility (Dana–Farber Cancer Institute, Boston).

44.22.11 is a rat monoclonal antibody specific for the murine V_β6 element (15). Monoclonal antibodies specific for I-$A^{b/d/p/q/r/k}$ (M5/114; Boehringer-Mannheim); CD1, CD4, CD8 (Becton Dickinson); and CD3 (Miles) were used at the final dilutions indicated.

Immunofluorescence. Cell surface immunofluorescence was quantitated using a fluorescence-activated cell sorter. Cells were incubated with monoclonal antibodies (final dilution 1:30) at 4°C for 40 min. Cells were then washed three times with phosphate-buffered saline (PBS) containing 2% (vol/vol) fetal calf serum and incubated with fluorescein-conjugated goat anti-rat IgG (1:300 dilution; Cappel Laboratories) for an additional 30 min before additional washing in PBS and analysis.

T-Cell Clones and Cell Proliferation Assays. O3 is a CD4+ type 1 T helper (T_H 1) clone derived from BALB/c mice after *in vitro* selection for proliferation to ovalbumin in association with BALB/c antigen-presenting cells (APCs) (13). To assay cell proliferative responses, 2×10^4 O3 cells were added to wells containing either 5×10^5 mitomycin-treated BALB/c spleen cells and OVA (10 μg/ml) or 5×10^5 mitomycin-treated DBA/2 or C57BL/6 spleen cells. In some experiments, splenic adherent cells were prepared by incubating 1×10^6 spleen cells after treatment with mitomycin C in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum for 2 hr before washing with a balanced salt solution containing 2% fetal calf serum to remove nonadherent cells (>90% of the total cell population). After 18–24 hr, each well was pulsed-labeled with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) and incubated for an additional 16 hr. Cultures were harvested with a Cell Harvester (Cambridge Technol-

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Abbreviations: APC, antigen-presenting cells; IFN-γ, interferon γ; RDU, relative densitometric unit(s); TCR, T-cell receptor; T_H, T helper; MHC, major histocompatibility complex; IL, interleukin.

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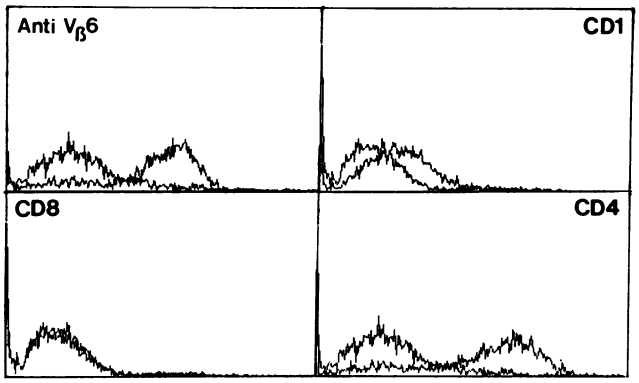

FIG. 1. Expression of cell surface products by O3 cells. Expression of CD1, CD4, CD8, and $V_\beta 6$ on O3 cells, as judged from indirect immunofluorescence using a panel of rat monoclonal antibodies to these determinants. The fluorescence-activated cell sorter profiles obtained for each antibody are shown and the intensity of fluorescence is presented in a $\log_{10}$ scale. Background fluorescence reflecting nonspecific binding of the second antibody was determined after incubation of cells with PBS instead of the relevant monoclonal antibody.

ogy, Cambridge, MA) and [^{3}H]thymidine incorporation was measured by liquid scintillation counting. The results are expressed as the mean of triplicate cultures.

Stimulation of O3 cells with immobilized anti-CD3 or anti-$V_\beta 6$ antibodies was performed in 96-well plates (Costar) that had been incubated overnight at 37°C with various concentrations of supernatant from either the 145-2C11 or the 44.22.11 hybridoma diluted in PBS (pH 8.4). After washing three times, each well received 2×10^4 O3 cells in a final volume of 0.2 ml of DMEM and 10% fetal calf serum.

Analysis of Cytokine Gene Expression. Cellular RNA was extracted using the guanidinium isothiocyanate method with a cesium chloride gradient modification (16). The amount of RNA in each sample was estimated by determining its absorbance at 260 nm (A_{260}). Samples (20 μg) of RNA from each cellular sample (unless otherwise indicated) were blotted onto nitrocellulose filters by using a Minifold II slot blotter (Schleicher & Schuell) and hybridized to a ^{32}P-labeled cDNA corresponding to the following genes: actin, *Eta-1* (a 1.6-kilobase *Hae* III fragment from pcD-Eta-1) (17), *GAPDH* (18), interleukin 2 (IL-2) (19), IL-3 (from pcD-IL3 isolated in our laboratory), or IFN-γ (20). Northern blot analysis showed that each IFN-γ cDNA probe hybridized to a single species of RNA and the levels of RNA quantitated by densitometric measurement of Northern blots were equivalent to those obtained by slot-blot analysis. Quantitation was performed as described (17) and detailed below. After preflashing, Kodak X-Omat films were exposed and the intensity of radioactivity of the autoradiograms was quantitated using an UltroScan II laser densitometer (LKB), adjusting exposure times so that the intensity of autoradiographic signals corresponded to the linear range of densitometric detection. To ensure that comparisons of cytokine RNA levels in different cellular samples were based upon the same amount of RNA in each sample, the area under the densitometric peak for cytokine RNA in each cellular sample was divided by the area under the actin or GAPDH densitometric peak for the same cellular RNA sample. The ratios of IFN-γ RNA to actin or GAPDH RNA for each cellular sample are expressed as relative densitometric units (RDU).

Table 1. Effect of monoclonal antibody 44.22.11 on the proliferative response of clone O3

Stimulus	[^{3}H]Thymidine incorporation, cpm $\times 10^{-3}$		
	Control	Anti-$V_\beta 6$	% reduction
BALB/c OVA/APCs	60.1	15.7	74
DBA/2	32.7	9.2	72
C57BL/6	33.4	13.8	59
IL-2	74.1	83.1	0

Cell cultures were incubated with monoclonal antibody 44.22.11 (anti-$V_\beta 6$) at a final dilution of 1:20. The proliferative response of O3 cells to mitomycin-treated OVA/APCs or DBA/2 or C57BL/6 spleen cells or to recombinant IL-2 (50 units/ml) was determined by [^{3}H]thymidine incorporation 24–36 hr after initiation of triplicate cultures; the standard error of the mean for each group was less than 12%. Addition of 44.22.11 had no effect upon the responses of two other $V_\beta 6^-$ CD4$^+$ T-cell clones (data not shown).

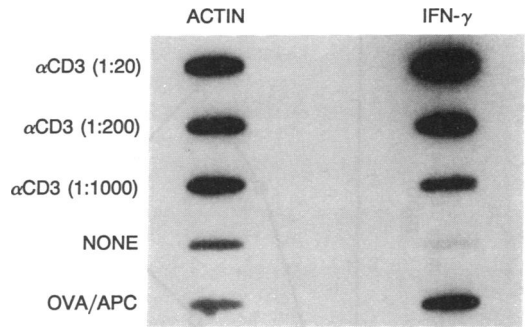

FIG. 2. Dose–response analysis of IFN-γ gene expression in O3 cells after stimulation by anti-CD3 antibody or protein antigen. O3 cells were incubated in wells that had been coated with the indicated final concentrations of anti-CD3 antibody. Two hours later cells were harvested for RNA extraction. Slot-blot analysis of IFN-γ and actin RNA from each cellular sample was performed and the resulting autoradiographic signals are compared with that of an RNA sample obtained from O3 cells 2 hr after stimulation with OVA-pulsed APCs (positive control).

RESULTS

Expression of $V_\beta 6$ on Clone O3 and Contribution to Antigen Reactivity. O3 is a T_H1 T-cell clone from BALB/c mice that was selected *in vitro* for responsiveness to ovalbumin in association with I-A^d (13). The clone also displays alloreactivity to I-A^b, possibly associated with a B-cell-specific peptide as well as responsiveness to an *Mls-1*^{a}-like determinant (14). Analysis of TCR β chain expression on this Thy-1$^+$CD4$^+$CD8$^-$ clone shows that it displayed $V_\beta 6$, as judged by immunofluorescence with the monoclonal antibody 44.22.11 (Fig. 1). Analysis of the effect of monoclonal antibody 44.22.11 on the three types of reactivity displayed by this clone (alloreactivity to IA^b, *Mls-1*^{a} reactivity, and reactivity to self MHC plus OVA) showed that this anti-$V_\beta 6$ antibody inhibited all three types of response but had no effect on the response to IL-2 (Table 1).

Cytokine Gene Expression After Activation of a T-Cell Clone by Various Stimuli. Ligation of the CD3 component of the TCR on O3 cells resulted in a dose-dependent induction of IFN-γ gene expression (Fig. 2). We compared IFN-γ gene expression after receptor ligation with either immobilized anti-CD3 or anti-$V_\beta 6$ antibody (Fig. 3). Although the anti-CD3 antibody stimulated IFN-γ gene expression more rapidly and efficiently than anti-$V_\beta 6$ antibody, ligation of the O3 TCR by both reagents was sufficient for induction of IFN-γ expression.

We then determined IFN-γ gene expression by O3 cells 4 hr after stimulation with OVA in association with syngeneic (BALB/c) spleen cells (OVA/APCs) or stimulation with DBA/2 spleen cells (*Mls-1*^{a}). The level of thymidine incorporation 24 hr after stimulation with either OVA/APCs or

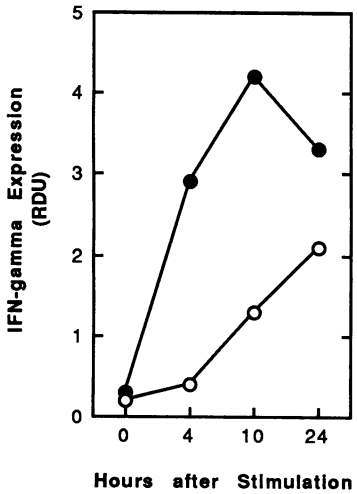

FIG. 3. Time course of IFN-γ gene expression after stimulation with anti-CD3 or anti-$V_\beta 6$ antibody. O3 cells were harvested at the indicated times after incubation in wells containing immobilized anti-CD3 (●) or anti-$V_\beta 6$ (○) antibodies that were applied to the plates at final dilutions of 1:10 for anti-CD3 and 1:2 for anti-$V_\beta 6$. The ratio of IFN-γ RNA levels to actin levels from each cellular sample as determined from densitometric analysis is expressed as RDU.

Mls-1^a was similar (Fig. 4). However, analysis of IFN-γ gene expression at 4 hr showed that although stimulation with OVA/APCs resulted in strong induction of IFN-γ gene expression, *Mls-1*^{a} stimulation was not accompanied by detectable induction of this gene.

In view of the similar levels of proliferation that accompanied the responses to OVA and *Mls-1*^{a}, we asked whether the apparent absence of IFN-γ gene expression 4 hr after activation of O3 cells by *Mls-1*^{a} reflected delayed rather than aborted IFN-γ gene expression. To this end, we measured IFN-γ gene expression at 4, 10, and 24 hr after initiation of the response to a protein antigen (OVA/APCs), an alloantigen (C57BL/6) or *Mls-1*^{a} (DBA/2) (Fig. 5). IFN-γ gene expression was induced 4 hr after activation with either protein antigen or alloantigen, reaching maximal levels of 20-fold and 6-fold elevation, respectively. However, no IFN-γ expression was detected 4, 10, or 24 hr after stimulation with *Mls-1*^{a} (DBA/2) spleen cells (Fig. 6). Despite these differences in cytokine gene expression, the level of

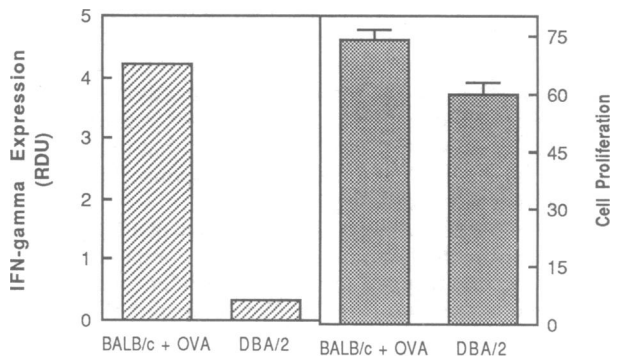

FIG. 4. Expression of IFN-γ after stimulation of O3 cells with OVA and syngeneic spleen cells or DBA/2 (Mls^a) spleen cells. Four hours after stimulation of O3 cells with mytomycin-treated BALB/c spleen cells plus 10 μg of OVA or mytomycin-treated DBA/2 spleen cells, O3 cells were harvested for RNA extraction. The level of IFN-γ gene expression (*Left*) and cell proliferation measured by [^{3}H]thymidine incorporation (cpm × 10^{-3}) 18–24 hr after the initiation of either culture (*Right*) are shown. Gene expression is shown as RDU of IFN-γ RNA compared with actin RNA in the same cellular sample.

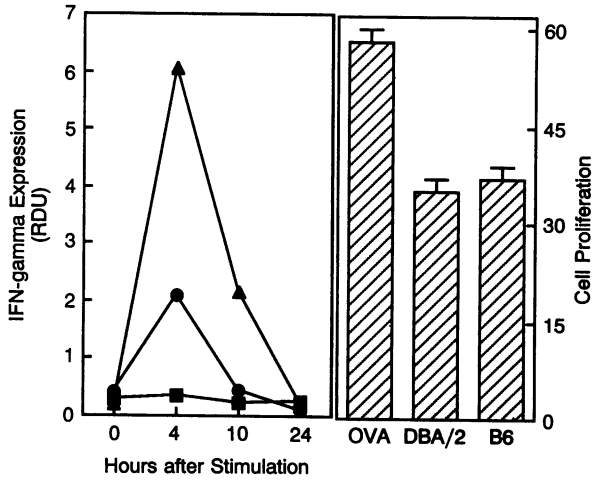

FIG. 5. Time course of IFN-γ gene expression after stimulation of O3 cells by various ligands. (*Left*) IFN-γ gene expression was determined at the indicated times after stimulation of O3 cells with BALB/c spleen cells plus 10 μg of OVA (▲), C57BL/6 spleen cells (●), or DBA/2 spleen cells (■). (*Right*) Cell proliferation measured as [^{3}H]thymidine incorporation (cpm × 10^{-3}) 18–24 hr after cultures were initiated is shown. Gene expression is shown as RDU of IFN-γ RNA compared with actin RNA in the same cellular sample.

proliferation after T-cell activation by the three stimuli was similar, as judged by thymidine incorporation between 18 and 24 hr (Fig. 5).

The defect in induction of IFN-γ gene expression after *Mls-1*^{a} stimulation shown above presumably reflected alterations in the signaling pathway that couples TCR ligation with intranuclear gene transcription. A recent study of T-cell activation has indicated that *Mls-1*^{a}-dependent stimulation is not accompanied by efficient phosphatidylinositol hydrolysis (21). In principle, this may lead to (*i*) decreased formation of diacylglycerol and a resultant decrease in activation of protein kinase C and/or (*ii*) decreased formation of inositol trisphosphates and reduced mobilization of intracellular calcium. The following observation suggests that a defect in calcium mobilization rather than protein kinase C activation is likely to be associated with impaired IFN-γ expression. Stimulation of O3 cells with phorbol 12-myristate 13-acetate, which artificially mimics intracellular diacylglycerol and activates protein kinase C, had no effect on IFN-γ expression

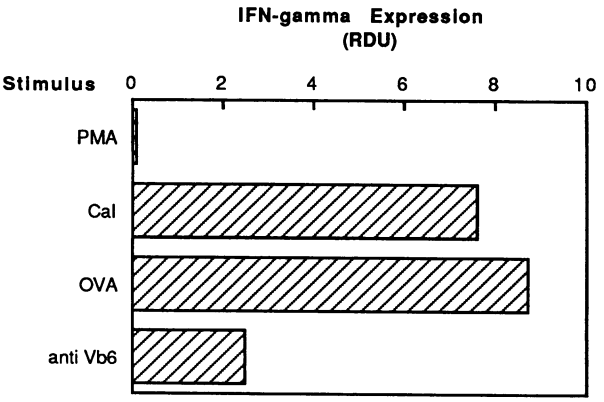

FIG. 6. IFN-γ gene expression after incubation with phorbol 12-myristate 13-acetate or the calcium ionaphore. O3 cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), the calcium ionophore (CaI) ionomycin (100 ng/ml), immobilized anti-$V_\beta 6$ antibody, or ovalbumin-pulsed splenic APCs (OVA) for 8 hr before harvesting cells and extracting RNA. Gene expression is shown as RDU of IFN-γ RNA compared with actin RNA in the same cellular sample. anti Vb6, anti-$V_\beta 6$.

(Fig. 6). In contrast, stimulation of O3 cells with the calcium ionophore ionomycin, resulting in increased levels of intracellular calcium, led to strong induction of IFN-γ gene expression (Fig. 6).

DISCUSSION

It has generally been assumed that engagement of the TCR by various ligands that efficiently stimulate T-cell proliferation results in similar patterns of cytokine gene expression. In this study, we have examined this assumption using a $CD4^+$ T_H1 clone that displays a strong proliferative response to the three general classes of T-cell ligands: peptide antigen, alloantigen, and *Mls-1*^{a} antigen. We found a clear disassociation between the cell's proliferative response and its ability to express the IFN-γ gene after engagement of its antigen receptor by the *Mls-1*^{a} ligand. These findings may be relevant to the unusual biological features of *Mls* responses. Although Mls-1^a antigens stimulate strong T-cell proliferative responses, they do not elicit efficient graft vs. host or homograft reactions *in vivo* (9) or generate cytoxic T-lymphocyte effector cells *in vitro* (22). This may, in part, reflect the failure of *Mls-1*^{a} antigens to stimulate the production of cytokines such as IFN-γ, which play an essential role in the development of these responses. By contrast, Mls recognition may enhance humoral immune responses. It has been shown (23) that the T-cell response to Mls markedly enhances induction of IgG antibody to cell surface antigens. The present studies suggest that enhancement may reflect the ability of Mls antigens to stimulate a cytokine response that lacks the B-cell inhibitory properties associated with IFN-γ (24–26).

These results also suggest that T-cell recognition of *Mls-1*^{a} may be qualitatively different than recognition of antigenic peptides and alloantigens. Both forms of recognition employ the V_β chain of the TCR, as judged by the inhibitory effects of anti-V_β6 antibody (Table 1). The finding is consistent with early studies of $CD4^+$ T-cell clones showing that expression of Mls^c reactivity was strongly correlated with the fine specificity of the TCR for protein antigen (27) and more recent work directly linking Mls reactivity with certain V_β elements (28, 29). A simple explanation for these observations is that the avidity of *Mls-1*^{a} recognition by the TCR is not sufficient to induce transmembrane and intracellular signals necessary for IFN-γ expression. However, preliminary studies of the functional avidity of O3 cells for the three ligands do not support this hypothesis. Alternatively, the interaction of Mls products with the TCR may be qualitatively different from the interaction of the TCR with conventional peptide antigen. Recent studies have indicated (30) that bacterial superantigens interact with MHC products outside the peptide-binding groove and may, therefore, engage segments of the TCR that are distinct from the peptide-binding site. This kind of collateral interaction may fail to ligate CD3 peptides that are necessary for transmission of intracellular signals, including, for example, phosphatidylinositol turnover (21), that may be essential for IFN-γ expression (see Figs. 2 and 3).

Expression of cytokines such as IFN-γ has been used to define functional heterogeneity within the $CD4^+$ T_H cell subset (31). We have not yet determined whether other cytokine genes, such as IL-4, may be expressed after *Mls-1*^{a} stimulation of this T_H clone. However, these studies suggest that a particular pattern of cytokine gene expression is not a stable phenotype of $CD4^+$ T_H cells and may be determined by the ligand responsible for receptor-mediated activation. They also open the possibility that stimulation of the same $CD4^+$ clone by conventional antigen or superantigen may transduce different intracellular signals leading to commitment to the T_H1 or T_H2 differentiation pathway and expression of alternative sets of cytokine genes.

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