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Immunology

Two monoclonal rat antibodies with specificity for the β-chain variable region $V_\beta 6$ of the murine T-cell receptor

(T-cell antigen receptor/mouse/T-cell clones)

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ABSTRACT **Two rat monoclonal antibodies (mAbs), 44-22-1 and 46-6B5, which recognize an alloreactive cytotoxic clone, 3F9, have been further tested on a panel of T hybridomas and cytotoxic T-cell clones for binding and functional activities. The mAbs recognized only those cells sharing the expression of the T-cell receptor β-chain variable region gene $V_\beta 6$ with 3F9. All $V_\beta 6^+$ cells were activated by these mAbs under cross-linking conditions and their antigen-specific activation was blocked by soluble mAb. Furthermore, depletion of 46-6B5$^+$ normal lymph node T cells eliminated all cells expressing the epitope recognized by 44-22-1 and $V_\beta 6$ mRNA.**

Several types of monoclonal antibodies (mAbs) and antisera have been raised against the α/β T-cell antigen receptor (TCR). One group of such antibodies consists of anti-clonotypic antibodies that are able to recognize the unique combination of an individual TCR α/β heterodimer (1–3). The second group includes antibodies that are specific for a single TCR protein chain—e.g., the mAb A2B4, which recognizes the TCR α chain of the T hybridoma (T Hy) 2B4 (4). A third and particularly useful class of TCR-specific reagents is composed of mAbs that are specific for individual TCR β-chain variable regions (V_β). This group includes KJ16 (5, 6) and F23.1 (7, 8), which recognize two or all members, respectively, of the three-membered $V_\beta 8$ family. Such antibodies have allowed analyses of V gene expression in different T-cell populations that was not possible with anticlonotypic reagents. Furthermore, the mAbs have been crucial tools in studies of thymic T-cell development. Another member of this group of reagents is the mAb KJ23, which recognizes $V_\beta 17$ (9). This mAb is of special interest, since correlation was found between KJ23 expression and IE^k alloreactivity.

Here we assign serological reagents to the third group; namely, mAbs that are specific for the murine TCR $V_\beta 6$ region. Two rat mAbs raised against the BALB/c-derived anti-$H\text{-}2^b$ cytotoxic T cell (T_c) 3F9 were originally described as anti-clonotypes (10). Further analyses with these antibodies, which we describe in this report, revealed that antibody binding to T cells correlates with expression of the $V_\beta 6$ gene segment of the TCR. Initial binding studies were confirmed functionally, proving that both mAbs are not anti-clonotypes but rather are specific for $V_\beta 6$, regardless of D_β (diversity), J_β (joining), or V_α expression.

MATERIALS AND METHODS

Cell Lines. All the cytotoxic and helper T-cell lines used in this investigation with their origin and specificities are described in Table 1. Interleukin 2 (IL-2)-dependent, mycoplasma-free CTLL-2 cells were obtained from American Type Culture Collection. A20, a B-cell lymphoma, was obtained from the same source.

Antibodies. Armenian hamster B hybridoma cells, 145-2C11, producing antibodies to mouse CD3 were a kind gift of J. Bluestone (19). The rat mAb hybridomas 44-22-1 (IgG2a) and 46-6B5 (IgM), which specifically inhibit T-cell clone 3F9, were produced by H. Acha-Orbea *et al.* (10). The rat mAb 9-1D10 is anti-mouse CD8 (10) specific. The mAb KJ16, which binds to TCR bearing either $V_\beta 8.1$ or $V_\beta 8.2$, but not $V_\beta 8.3$ (5, 6), was obtained from J. Kappler and P. Marrack (National Jewish Hospital, Denver). Fluorescent antibodies included fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-rat IgG (heavy and light chain specific) and FITC-conjugated affinity-purified goat anti-hamster IgG (heavy and light chain specific) $F(ab')_2$ fragments from Cappel Laboratories (Cooper Biomedical, Scientific Division, West Chester, PA), as well as FITC-conjugated goat anti-rat immunoglobulin (absorbed on mouse immunoglobulin) from Tago (Burlingame, CA).

FACS Analysis. *T Hy.* Aliquots of 10^6 cells in 100 μl were stained with 44-22-1 or 145-2C11 as neat hybridoma culture supernatants in 0.1% NaN_3 followed by fluorescent goat anti-rat (44-22-1) or anti-mouse (145-2C11) immunoglobulin labeling. Flow cytometric analysis was carried out with a Becton-Dickinson FACScan System.

T_c clones and normal lymph node cells. Aliquots of 10^6 cells in 100 μl were stained at 4°C with 44-22-1, 46-6B5, or KJ16 rat mAb as 25% hybridoma culture supernatants followed by a fluorescent goat anti-rat immunoglobulin labeling. Flow cytofluorometric analysis of viable cells was done on either a Becton-Dickinson FACS II and IV or an Ortho Cytofluorograph 50.H instrument.

Data for both groups are expressed as mean fluorescence intensities in arbitrary units after analyzing 10^4 cells.

Activation with mAbs. *Sepharose-coupled mAb.* Rat mAbs were purified by ammonium sulfate precipitation of ascites fluid and coupled to CNBr-Sepharose 4B beads following the manufacturer's protocol (Pharmacia, Uppsala). Two × 10^4 cloned cytotoxic T lymphocytes in 0.2 ml of complete tissue culture medium in the absence of IL-2 were incubated with 10^3 mAb-modified Sepharose beads. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) to measure proliferation of the cells.

FcR crosslinking. Cultures containing 4 × 10^4 T Hy and 10^6 A20 cells bearing FcR were incubated with and without mAb as 25% hybridoma culture supernatants in a total vol of

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Abbreviations: T Hy, T cell hybridoma; T_c, cytotoxic T cell; TCR, T-cell antigen receptor; mAb, monoclonal antibody; IL-2, interleukin 2; FITC, fluorescein isothiocyanate; V, variable; J, joining; D, diversity; C, constant.

Table 1. Specificity of TCR genes by T-cell clones in correlation with mAb binding

T-cell clone or hybridoma	Mouse strain	Specificity	T cell type	TCR		mAb binding		
				α chain	β4 chain	44-22-1	46-6B5	Ref.
3F9 (3A2)	BALB/c	Alloreactive D^b	T_c	V_α8.3F9 J_αTA19	V_β6 J_β1.1	+	+	10, 11
LB2-1	B6	I-A^b-CRC (Mls^a)	T_H	V_α8.3F9 J_αLB2	V_β6 D_β J_β2.1 J_β2.3	+	+	12, 13
5.3.18	bm12	bm12-insulin-B	T_H	V_α42H11.2	V_β6 D_β2.1 J_β2.3	+	+	14, 15
42F7	bm12	bm12-Auto	T_H	Not V_α3 or V_α AF.3.G7	V_β6	+	+	14, 15
C9	B6	D^b AED	T_c	V_α3.C9 J_αC9	V_β6 D_β1 J_β1.1	+	+	16, 17
653	BALB/c	Alloreactive D^b	T_c	V_α4.3.1 J_α653	V_β6 D_β1 J_β2.6	+	+	†
24	BALB/c	Alloreactive D^b	T_c	ND	V_β6 (rearranged)	+	+	†
25	BALB/c	Alloreactive D^b	T_c	ND	V_β6 (rearranged)	+	+	†
5/10-20(K)	B6	K^b AED	T_c	V_α8.520K J_α520K	V_β7 D_β1.1 J_β1.2	−	−	16, 17
5/10-20(D)	B6	D^b AED	T_c	V_α1.520D J_α810	V_β5.2 D_β1.1 J_β2.6	−	−	16, 17
52H10	B6	B6-insulin-A	T_H	V_αAF3.G7*	V_β4 D_β2 J_β2.7	−	−	14, 15
10.10.58	bm12	bm12-insulin-B	T_H	Not V_α3 or V_αAF3.G7	Not V_β4 or V_β6	−	−	14, 15
42H11	bm12	bm12-insulin-A	T_H	V_α42H11.1	V_β4 D_β2 J_β2.7	−	−	14, 15

T_H, helper T cells; ND, not done.
*V_α52H10 is identical by partial sequencing and restriction enzyme mapping to V_αAF3.G7, a V_α3 family member (18).
†M.W.S., unpublished data.

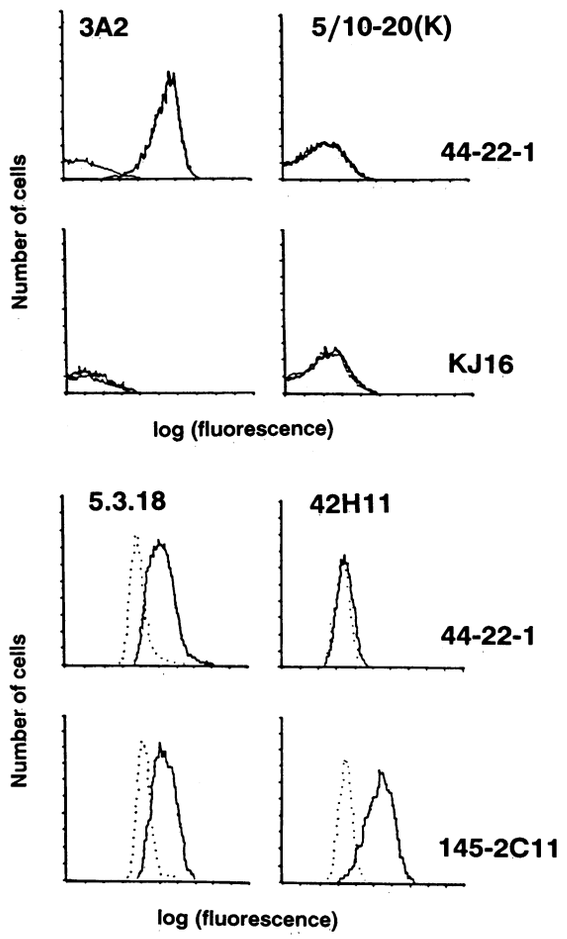

FIG. 1. (*Upper*) Binding of 44-22-1 and KJ16 to 3A2 and 5/10-20(K). The two T_c clones 3A2 (V_α8.3F9, V_β6) and 5/10-20(K) (V_α8.520K, V_β7) were cytofluorometrically analyzed after labeling with the rat mAb 44-22-1 or KJ16 (—) followed by FITC-conjugated goat anti-rat IgG antibodies. —, Staining with the fluorescent second antibody alone. (*Lower*) Binding of 44-22-1 and 145-2C11 to 5.3.18 and 42H11. The two T Hy 5.3.18 (V_α3, V_β6) and 42H11 (V_α3, V_β4) were cytofluorometrically analyzed after labeling with 44-22-1 or 145-2C11 (—) followed by FITC-conjugated goat anti-rat (44-22-1) or anti-hamster (145-2C11) immunoglobulin antibodies. ····, The fluorescent antibody alone.

0.2 ml. The mAb included 44-22-1 and 46-6B5 as test mAb and 145-2C11 as a positive control. Secreted IL-2 levels were measured after 24 hr by incubating IL-2-dependent CTLL-2 cells (6×10^3) with 50 μl of cell-free culture supernatant, followed by 1 μCi of [^{3}H]thymidine at 6 hr, and harvesting after 18 hr with a multiautomated harvester. Incorporation of radioactivity is expressed as means measured from triplicate samples.

Blocking of Antigen Response with 46-6B5. Antigen-specific activation of T Hy was measured by a standard IL-2 assay method (20). Cultures containing $3–4 \times 10^4$ T Hy and 5×10^5 irradiated spleen cells as antigen presenting cells were incubated with or without various doses (12.5–800 μg/ml) of antigen (beef insulin) for 24 hr in a vol of 0.2 ml. Secreted IL-2 was measured as described above. Blocking of antigen-stimulated IL-2 production was tested by including the mAb 46-6B5 as hybridoma culture supernatant at various concentrations in the 0.2-ml IL-2 production cultures described above. Incorporation of radioactivity was measured and is expressed as means of triplicate samples.

Depletion of Lymph Node Cells Using mAb 46-6B5 and Complement. Nylon wool purified BALB/c lymph node cells were treated twice with mAb 46-6B5 as 50% culture supernatant together with 5% rabbit complement (Cedarlane Laboratories, Hornby, ON) for 30 min at 37°C. Untreated and 46-6B5-depleted cells were stimulated with phorbol 12-myristate 13-acetate (3 ng/ml), ionomycin (250 ng/ml), and recombinant human IL-2 (rIL-2) (30 units/ml) at a concentration of 2.5×10^5 cells in complete tissue culture medium. Cultures were diluted with fresh medium containing 20 units of rIL-2 per ml on day 4 and were harvested on day 7.

Table 2. [^{3}H]Thymidine uptake after stimulation with crosslinked mAb

T Hy	Relative IL-2 production induced by mAb crosslinked via FcR (specificity)		
	Medium	46-6B5 (αV_β6)	145-2C11 (αCD3)
5.3.18	944	9894	26,353
42H11	817	2907	30,776
52H10	636	771	20,238

Cultures (0.2 ml) containing T Hy and 1×10^6 mitomycin C-arrested A20 cells were incubated without and with mAb (46-6B5, 50% culture supernatant; 145-2C11, 25% culture supernatant). Secreted IL-2 was measured as described.

Table 3. $V_\beta 6$-specific inhibition of insulin-specific activation of T Hy by mAb 46-6B5

	Relative IL-2 production ([^{3}H]thymidine uptake)				
	mAb 46-6B5 (IgM) + insulin				
T Hy	None	12.5%	25%	50%	No insulin
5.3.18	10,460	6438	3170	1079	690
42H11	8,286	7594	7273	7453	681
52H10	4,107	4225	4249	4393	542

T Hy were incubated in the presence of irradiated splenocytes, ±500 μg of beef insulin per ml (Sigma), at the indicated final concentration of mAb 46-6B5 in percentage of hybridoma supernatant. Levels of secreted IL-2 were measured as described.

Aliquots of the cells were stained either with mAb 44-22-1 or KJ16 followed by FITC-conjugated goat anti-rat immunoglobulin for FACS analysis.

RNA Blot Analysis. Total cellular RNA was prepared by lysis of cells in 1% Nonidet P-40 and subsequent phenol extraction as described by Scott *et al.* (21). RNA was fractionated by electrophoresis in a glyoxylate-buffered agarose gel (22) and transferred onto Hybond-N nylon membrane (Amersham).

Filters were hybridized with ^{32}P-labeled nick-translated DNA probes in 50% formic acid/5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/50 mM potassium phosphate buffer, pH 6.5/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/1% sodium dodecyl sulfate (SDS)/denatured salmon sperm DNA (0.1 ng/ml) at 42°C. Filters were washed twice for 10 min in 2× SSC/0.1% SDS at 42°C and once in 0.3× SSC/0.1% SDS at 42°C for 30 min. The DNA fragments were $V_\beta 6$, an *Eco*RI/*Xho* I endonuclease fragment of the cDNA clone 3F9β1 (11). Filters were washed twice in water at 100°C for 5 min for further hybridization experiments.

RESULTS AND DISCUSSION

Analysis of the Fine Specificity of the mAb 44-22-1 and 46-6B5. The mAb 44-22-1 and 46-6B5 were derived from a fusion of the myeloma cell line Y3M and spleen cells from a Lou rat immunized with the alloreactive T_c clone 3F9 (10). They were selected based on their capacity to inhibit the cytotoxic activity of 3F9. They also reacted with the chicken erythrocyte-specific IA^b-restricted helper T-cell clone LB2-1 that shares variable region genes with 3F9—namely, $V_\alpha 8$ and $V_\beta 6$—but expresses different D and J region segments (12, 13). To further characterize the specificity of the two mAb, they were screened on a panel of helper T Hy and T_c clones. Origin, antigen specificity, H-2 restriction, function, and usage of V_α and V_β TCR gene segments of these cells are listed in Table 1. Both mAb specifically bound to all T cells that express $V_\beta 6$ irrespective of V_α, D_β, or J_β segment usage. Specificity for $V_\beta 6$ was further confirmed by the negative results obtained with the T-cell clone 5/10-20(K) that expresses the identical V_α gene segment to 3F9 and LB2-1, but a different V_β, and the T Hy 42H11, which expresses a V_α gene 98% similar to the V_α utilized by 5.3.18 and $V_\beta 4$. Fluorescence histograms for both pairs of cells are shown in Fig. 1. All the control T-cell lines described in Table 1 were negative in FACS analyses with 44-22-1 and 46-6B5 (data not shown). Reciprocal competitive antibody binding studies with 46-6B5 and 44-22-1 suggest that the two mAbs recognize sterically very closely located epitopes on the TCR protein (R.S., unpublished results).

Functional Activation and Inhibition of T Hybridomas and T-Cell Clones by mAb 44-22-1 and 46-6B5. As several groups have described for other anti-TCR reagents (1, 3, 13, 14), we found that serological crosslinking of the TCR complex via the FcR on a bystander cell or by mAb coupled to Sepharose beads specifically activated $V_\beta 6^+$ T Hy and T-cell clones, as shown in Table 2. In these experiments, the $V_\beta 6^+$ T Hy 5.3.18 was specifically triggered by 46-6B5 in the presence of FcR-bearing A20 cells. The $V_\beta 4$–TCR α-matched T Hy, 42H11, was not activated by 46-6B5, nor were the 52H10 or 10.110 T Hy, which have very similar specificities to 5.3.18. Each of the T Hy could be activated through the TCR complex by using the murine CD3-specific mAb 145-2C11. Isotype-matched control antibodies to 44-22-1 did not activate any of the T Hy (results not shown). The $V_\beta 6^+$ T_c clone 3F9 was activated by 44-22-1 and 46-6B5 coupled to Sepharose (results not shown).

An identical pattern could be derived for the blocking capacity of 46-6B5. As shown in Table 3, binding of the mAb specifically interfered with antigen recognition by the TCR in the $V_\beta 6^+$ T Hy 5.3.18. The mAb 46-6B5 blocked the IL-2 response of this T Hy to insulin very efficiently, while insulin responses of $V_\beta 6$-42H11 and 52H10 were not altered.

44-22-1 and 46-6B5 mAb Recognize All $V_\beta 6^+$ TCR Expressing Normal T Lymphocytes. To correlate the expression of the epitopes recognized by mAb 44-22-1 and 46-6B5 and the presence of $V_\beta 6$ mRNA we performed T-cell depletion experiments. Normal lymph node cells were treated with 46-6B5 plus complement and subsequently nonspecifically stimulated to proliferate. After 7 days, aliquots of the 46-6B5 depleted and control cell cultures were cytofluorometrically

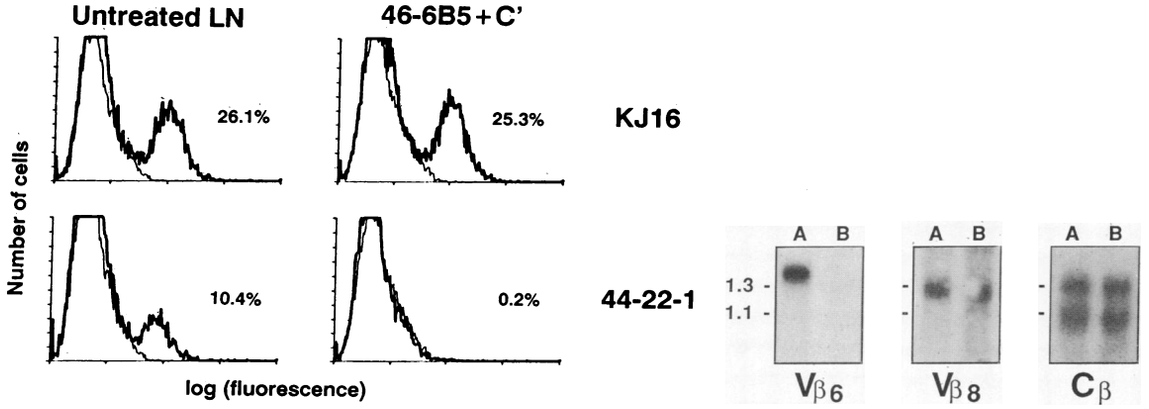

FIG. 2. (*Left*) Depletion of $V_\beta 6^+$ T cells by rat mAb 46-6B5 plus complement treatment. Lymph node cells (untreated and treated with 46-6B5 plus complement) were labeled with 44-22-1 or, as a control, with KJ16 (anti-$V_\beta 8$) and FITC-conjugated goat anti-rat IgG antibodies. Fluorescence analysis was carried out on a FACS II flow cytometer. (*Right*) RNA blot analysis of the total cellular RNA extract of untreated and 46-6B5 plus complement-treated lymph node cells was performed by using radioactive $V_\beta 6$, $V_\beta 8$, and C_β TCR probes.

analyzed with the rat mAb KJ16 and 44-22-1 and their cytoplasmic RNA was extracted. As shown in Fig. 2 (*Left*), the mAb 44-22-1 stains ≈10% of normal lymph node cells. Depletion of the 46-6B5$^+$ cells resulted in the complete loss of 44-22-1$^+$ cells, while the percentage of KJ16$^+$ T cells remained unchanged. These results strongly suggest that the 44-22-1$^+$ lymphocytes are identical to those expressing the epitope recognized by 46-6B5. Furthermore, RNA blot analysis of RNA extracted from these cells with $V_\beta 6$, $V_\beta 8.2$, and C_β (constant region) probes revealed that the mAb 46-6B5$^+$ cells depleted cultures selectively lacked $V_\beta 6$ mRNA. In contrast, hybridization with $V_\beta 8.2$ and C_β probes resulted in signals of proportional intensities. These data demonstrate directly that all normal T lymphocytes utilizing the $V_\beta 6$ gene segment are recognized by mAb 44-22-1 and 46-6B5. However, the possibility that these mAb recognize some T lymphocytes using other V_β genes (with the exception of the $V_\beta 8$ family) cannot be formally excluded (although no such cells have yet been detected in our analysis of T-cell clones).

Conclusions. Our results clearly demonstrate that the mAbs 44-22-1 and 46-6B5 recognize not the TCR clonotype of 3F9 but rather an epitope(s) expressed by the $V_\beta 6$ region, since both mAb specifically bind to and functionally affect all $V_\beta 6^+$ T Hy and T-cell clones regardless of the combinatorial rearrangements of the remaining TCR gene segments. This finding was confirmed in whole T-cell population studies since we found that all $V_\beta 6$ expressing normal lymph node T cells were eliminated by mAb 46-6B5 and complement treatment. These mAb will further facilitate analysis of the acquisition and expression of the T-cell receptor repertoire.

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1. Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J. W. & Marrack, P. (1984) *J. Exp. Med.* **157**, 1149–1169.
2. Allison, J. P., McIntyre, B. W. & Bloch, D. (1982) *J. Immunol.* **129**, 2293–2300.
3. Meuer, S. C., Fitzgerald, K. A., Hussey, R. E., Hodgdon, J. C., Schlossman, S. F. & Reinherz, E. L. (1983) *J. Exp. Med.* **157**, 705–719.
4. Saito, T., Weiss, A., Miller, J., Norcross, M. A. & Germain, R. N. (1987) *Nature (London)* **325**, 125–130.
5. Behlke, M. A., Henkel, T. J., Anderson, S. J., Lan, N. C., Hood, L., Braciale, V. L., Braciale, T. J. & Loh, D. Y. (1987) *J. Exp. Med.* **165**, 257–262.
6. Haskins, K., Hannum, C., White, J., Roehm, N., Kubo, R., Kappler, J. W. & Marrack, P. (1984) *J. Exp. Med.* **160**, 452–471.
7. Staerz, U. D., Rammensee, H. G., Benedetto, J. D. & Bevan, M. J. (1985) *J. Immunol.* **134**, 3994–4000.
8. Kappler, J. W., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, N. & Marrack, P. (1987) *Cell* **49**, 263–271.
9. Kappler, J. W., Roehm, N. & Marrack, P. (1987) *Cell* **49**, 273–280.
10. Acha-Orbea, H., Zinkernagel, R. M. & Hengartner, H. (1985) *Eur. J. Immunol.* **15**, 31–36.
11. Rupp, F., Acha-Orbea, H., Hengartner, H., Zinkernagel, R. M. & Joho, R. (1985) *Nature (London)* **315**, 425–427.
12. Giedlin, M. A., Longenecker, B. M. & Mosmann, T. R. (1986) *Cell. Immunol.* **97**, 357–370.
13. Rupp, F., Brecher, J., Giedlin, M. A., Mosmann, T., Zinkernagel, R. M., Hengartner, H. & Joho, R. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 219–222.
14. Hochman, P. S. & Huber, B. T. (1984) *Eur. J. Immunol.* **14**, 610–615.
15. Sherman, D. H., Hochman, P. S., Dick, R., Tizard, R., Kuzhalmannam, R. L., Flavell, R. A. & Huber, B. T. (1987) *Mol. Cell. Biol.* **7**, 1805.
16. Pircher, H. P., Hämmerling, G. J. & Hengartner, H. (1984) *Eur. J. Immunol.* **14**, 144–152.
17. Iwamoto, A., Ohashi, P. S., Pircher, H. P., Walker, C., Michalopoulos, E., Rupp, R., Hengartner, H. & Mak, T. W. (1987) *J. Exp. Med.* **165**, 581–600.
18. Spinella, D. G., Hansen, T. H., Walsh, W. D., Behlke, M. A., Tillinghast, V. P., Chou, H. S., Whiteley, P. J., Kapp, J. A., Pierce, C. W., Shevach, E. M. & Loh, D. Y. (1987) *J. Immunol.* **138**, 3991–3995.
19. Oberdan, L., Foo, M., Sachs, D. H., Samuelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1374–1378.
20. Heber-Katz, E., Schwartz, R. H., Matis, L. A., Hannum, C., Fairwell, T., Apella, E. & Hansburg, D. (1982) *J. Exp. Med.* **55**, 1086–1090.
21. Scott, M. R. D., Westphal, K. H. & Rigby, P. W. J. (1983) *Cell* **34**, 557–567.
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), p. 200.