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Human T-Cell Responses to *Mycoplasma arthritidis*-Derived Superantigen

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When injected into mice, *Mycoplasma arthritidis* causes a chronic arthritis that resembles rheumatoid arthritis, histologically. The organism produces a superantigen termed *Mycoplasma arthritidis* mitogen or MAM, that in humans preferentially expands T cells whose antigen receptors express Vβ 17. T cells with this phenotype appear to be increased in rheumatoid synovial effusions. We describe a novel approach to isolating and characterizing human MAM-reactive T-cell lines and determining their T-cell receptor (TCR) Vβ usage. Lines were prepared from T cells that clustered with dendritic cells during a 2-day exposure to MAM. Cluster and noncluster fractions of T cells were then expanded by using feeder cells and a polyclonal mitogen. Most of the MAM reactivity was found in dendritic T-cell clusters, as were most of the T cells expressing TCR Vβ 17. After expansion, 76% of the cluster-derived T-cell lines were MAM reactive, while no reactivity was seen in cell lines derived from the noncluster fraction. Of the MAM-reactive lines, 49% expressed Vβ 17 on some or all of the cells. Cell lines from both cluster and noncluster fractions were analyzed for TCR Vβ mRNA expression by PCR amplification. Other Vβ genes (5.1, 7, 8, 12, and 20) were found to be expressed by lines that were MAM reactive, although these were not a major component of the cluster-derived T cells. Some non-cluster-derived lines expressed Vβs 17, 12, and 7, but these proved to be nonreactive to MAM. Therefore, dendritic cells can be used to immunoselect and characterize T cells that express superantigen-reactive TCRs.

Superantigens (SAgs) are proteins produced by certain bacteria, mycoplasmas, and viruses (13, 22, 31). They differ from conventional antigens in several ways. SAgs do not require processing by antigen presenting cells (APCs) but instead bind directly and with high affinity to class II molecules at sites outside the peptide binding groove (22). Upon presentation by major histocompatibility complex (MHC) class II, SAgs interact with the cell surface products of subsets of T-cell receptor (TCR) Vβ genes. SAgs are potent mitogens for T cells expressing the appropriate Vβs, often stimulating >5% of the total lymphocyte pool (reviewed in reference 31). Many of the proteins with SAg activity cause a variety of diseases in both humans and animals. These diseases are typically acute, e.g., enterotoxins derived from *Staphylococcus aureus* induce gastroenteritis and the toxic shock syndrome (31, 38), ascribed to the massive release of cytokines from the stimulated T cells (32, 42).

Mycoplasma arthritidis produces a SAg (*M. arthritidis* mitogen [MAM]) which has been implicated in the induction of a chronic polyarthritis that develops following infection of mice (10). The arthritis has many features resembling rheumatoid arthritis (RA) histologically (13, 14). The joint disease that is induced in mice by the intravenous injection of viable organisms is actually preceded by a toxic syndrome resembling that induced by the staphylococcal SAgs (2). The syndrome appears to be dependent on MAM, since it is mimicked by injection of this SAg, and toxicity occurs only in mice that express the I-E molecule to which MAM appears to bind preferentially (10). Intraarticular injection of MAM into rats can induce a severe but transient synovitis, implying that MAM also contributes to joint inflammation mediated by live organisms (10).

MAM has several intriguing properties. Most importantly, it specifically expands T cells bearing TCR Vβ gene products that have been implicated in murine models of arthritis (Vβ 6 and 8 [10]) and in patients with RA (Vβ 17 [19, 23]). Moreover, MAM may be more potent than other SAgs in inducing polyclonal immunoglobulin production, including rheumatoid factors by human mononuclear cells (15, 40). To address the possibility that this agent has a potential role in the pathogenesis of RA, it would be useful to develop panels of human T-cell clones or lines that are well characterized with respect to their MAM reactivity and TCR usage. The lines would be potentially important in screening joint APCs from rheumatoid patients for the presence of MAM or MAM-related proteins.

In this study, dendritic cells exposed to MAM were used to generate MAM-reactive T-cell lines which were then analyzed for reactivity and for Vβ expression. We find that dendritic cells can efficiently select MAM-reactive T cells into clusters and that most of the MAM-reactive cells are Vβ 17 positive. In addition, other TCR gene products expressed by some MAM-responsive T cells have been identified. The noncluster fraction contains rare cells which, upon expansion, express the same TCR Vβ as the cluster-derived lines, but the former are not MAM reactive.

MATERIALS AND METHODS

Culture medium. RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 5% human serum, 50 μg of gentamicin per ml and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was used throughout these studies except as indicated.

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Preparation of lymphoid cells. A $DR4^+$ (heterozygous) normal donor served as the source of blood mononuclear cells from which MAM-reactive T-cell lines were developed. Mononuclear cells were also purified from buffy coats obtained from the New York Blood Center. They served as the source of peripheral blood mononuclear cells for phenotyping studies (see below) and feeder cells for maintenance of the cell lines. Mononuclear cells were separated into T-cell-enriched (ER^+) and T-cell-depleted (ER^-) fractions by rosetting with neuraminidase-treated sheep erythrocytes as previously described (4).

T cells. ER^+ cells were depleted of non-T-cell contaminants first by panning on petri dishes coated with human gamma globulin to remove FcR^+ cells (44) and then coating MHC class II-positive cells with anti-DR/DQ monoclonal antibody (MAb) 9.3C9 (immunoglobulin G2a [IgG2a], subclone of HB180; American Type Culture Collection, Rockville, Md.) followed by panning on petri dishes coated with goat-anti-mouse IgG (Cappel Laboratories, Organon Teknika, Durham, N.C. [17, 45]). The resulting T-cell preparations failed to proliferate to optimal doses of SAgs, indicating negligible contamination with APCs.

APC populations. The ER^- cells were used as feeder cells for growth of the T-cell lines. To obtain partially enriched populations of dendritic cells, the ER^- or T-depleted fraction was depleted of residual monocytes by panning on gamma globulin-coated plates to yield ER^- FcR^- populations. These generally contain 5% dendritic cells. For further purification of dendritic cells, the ER^- FcR^- cells were layered onto 14.5% metrizamide (18). After sedimentation at $650 \times g$ for 10 min, B cells and a few natural killer cells localize to the high-density pellet, while dendritic cells are enriched at the low-density interface. The purity of dendritic cells ranged from 50 to 75%, with contaminants being primarily small B cells and natural killer cells.

SAgs. MAM was prepared by a new protocol based on that previously described (2). In brief, $[NH_4]_2SO_4$ fractionation was followed by Sephadex G-50 chromatography and subsequent passage over Sepharose and Mono S ion-exchange columns. The resulting material was about 20% pure and contained <50 μg/ml (11).

Enrichment of MAM-reactive T cells into dendritic cell–T-cell clusters. We have previously shown that highly enriched sources of dendritic cells (metrizamide gradient low-density cells [see above]) pulsed with antigen have the ability to cluster antigen-reactive T cells into discrete units (34, 35). A partially enriched preparation of dendritic cells (ER^- FcR^- cells [see above]) also suffices in this regard. A total of 1×10^6 T cells were cultured with 2×10^5 ER^- FcR^- cells (5:1 ratio) in the presence of MAM at a final dilution of 1:4,000, in 1 ml of RPMI containing 5% human serum in flat-bottomed 16-mm well plates (Costar, Cambridge, Mass.). The MAM dose used corresponded approximately to <10 ng/ml. At the end of the culture period, generally 36 to 48 h, clusters of dendritic cells and responding T cells develop, as previously described (17, 34, 35). The clusters were separated from the nonclustered cells by application over 30% fetal calf serum gradients. After 2 h of $1 \times g$ sedimentation at 4°C, the clustered T cells moved to the bottom while the nonclustered T cells were harvested from the top of the gradient. Clusters were then cultured in 16-mm wells for an additional hour to allow the release of loosely adherent T cells, after which the clusters were reapplied to 30% serum gradients.

Generation of MAM-reactive T-cell lines. T-cell lines were generated from clustered and nonclustered cells by seeding at a limiting dilution (1 or 10 cells per well) in round-bottom 96-well microtiter plates, immediately after their isolation. Feeder cells were buffy coat-derived ER^- cells. These were treated with mitogen (sodium periodate) (34, 35), irradiated with 3,000 rads [^{137}Cs], and added at 10^4 cells per well. Control plates were seeded with T cells alone or stimulator cells alone. Interleukin-2 (IL-2; 15% [vol/vol]) (Pharmacia, ENI, Silver Springs, Md.) was added to all wells. T-cell lines become apparent about 10 days after seeding. Once visualized, the cell lines were immediately tested for MAM reactivity and then reexpanded for evaluation of their TCR phenotype (see below). The lines were maintained long term with periodate-treated allogeneic feeder cells and IL-2. The majority of lines obtained were $CD4^+$ (data not shown).

Lymphocyte proliferation assays. (i) SAg specificity of the clustered and nonclustered T cells. Bulk, clustered, and nonclustered T cells were collected on the second day of culture with MAM and partially enriched preparations of dendritic cells. The populations were cultured an additional 3 days and then added in graded numbers to APCs (syngeneic ER^- cells) and MAM at a final concentration of 1:4,000. The cultures were pulsed at 72 h with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) for 14 h.

(ii) SAg specificity of the clustered and nonclustered T-cell lines. T-cell lines that were generated from cluster and noncluster fractions were tested immediately for MAM reactivity. The cells were washed to remove residual IL-2 as previously described (35) and then rechallenged with APCs (syngeneic ER^- cells) in the presence or absence of MAM. The cultures were pulsed at 72 h with 1 μCi of [^{3}H]TdR for 14 h. Results are expressed as stimulation indices (SI), where SI = counts per minute of T-cell line + APC + MAM/counts per minute of T-cell line + APC.

Phenotyping. Bulk, clustered, and nonclustered T cells and T-cell lines were analyzed for their expression of CD3, CD4, CD8, and TCR usage with a panel of MAbs by indirect immunofluorescence. Antibodies directed to the T-cell surface antigens were CRL 8001 (anti-CD3), FFB 2.3 (anti-CD4 [39]), and CRL 8014 (anti-CD8). Anti-TCR V region MAbs were C1 (anti-Vβ 17 [19]), S511 (anti-Vβ 12 [8]), Ti3a (anti-Vβ 8 [1]), OT145 (anti-Vβ 6.7a [28, 37]), LC4 (anti-Vβ 5.1 [30]), and C37 (anti-Vβ 5.2/5.3 [41]). They were used at 50 μl of supernatant per stain. Isotype controls were MAbs with irrelevant specificities: 44.3, IgG1, anti-idiotype MAb to a human IgM kappa chain; IgG2a, anti-DNP MAb; and IgG2b, myeloma protein.

Analysis of TCR Vβ gene usage by PCR. T-cell lines were grown to approximately 10^7 cells, and RNA was extracted with RNAzol (Biotecx Labs Inc.) according to the manufacturer's instructions. cDNA was synthesized with murine reverse transcriptase and a poly(dT) primer, as previously described (19, 29). Optimal amounts of cDNA (determined in preliminary experiments) were used as a template in parallel PCRs in which 23 different Vβ-specific sense primers (Table 1) were each paired with a common antisense Cβ-specific primer. The PCR mixture included 1.5 mM $MgCl_2$, 2 U of *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 0.1% (wt/vol) gelatin in a 50-μl volume. Each PCR cycle was set at 94°C for 60 s, 51°C for 60 s, and 72°C for 90 s for 28 cycles, with a final extension cycle at 72°C for 10 min. Ten microliters of PCR product was then run on a 1.5% agarose gel and visualized by ethidium bromide staining. Each experiment also included a positive control amplification from a Vβ 6.7a containing plasmid with Vβ 6 and Cβ primers. Ten microliters of this PCR product

TABLE 1. Vβ-specific sense primers for PCR[a]

Vβ gene	Sequence (5'–3')
Vβ 1	GCACAACAGTTCCCTGACTT
Vβ 2	GTTTCTCATCAACCATGCAA
Vβ 3	CTAGAGAGAAGAAGGAGCGC
Vβ 4	ATGAGAGGGATTTGTCATTG
Vβ 5.1/5.4	AGTGAGACACAGAGAAACAAA
Vβ 5.2/5.3	GTCAGGGGCCCCAGTTTAT
Vβ 6	TCAGGTGTGATCCAATTTC
Vβ 7	CCTGAATGCCCCAACAGCT
Vβ 8.1/8.2	ATTTACTTTAACAACAACGTT
Vβ 9	CCTAAATCTCCAGACAAAGC
Vβ 10	TCAATGAGCGATTTTTAGCC
Vβ 11	TCAACAGTCTCCAGAATAAG
Vβ 12	CAAAGGAGAAGTCTCAGAT
Vβ 13.1	TGCTGGTATCACTGACCAA
Vβ 13.2	GGTGAGGGTACAACTGCC
Vβ 14	CTCGAAAAGAGAAGAGGAATT
Vβ 15	GTCTCTCGACAGGCACAGGCT
Vβ 16	GTCTAAACAGGATGAGTCC
Vβ 17	ACAGCGTCTCTCGGGAGA
Vβ 18	GAAAATATCATAGATGAGTCA
Vβ 19	ACAAGTTCTTCAAGAAACGG
Vβ 20	GTATTGACCAGATCAGCTCT
Cβ (antisense)	CTTCTGATGGCTCAAACAC

[a] The amplified segment will vary depending upon the length of the VDJ junction but should be approximately 200 to 350 bp. These primers are similar to those used by Choi et al. (9).

was run on both sides of each agarose gel serving as a positive control for the PCR and as a size marker (326 bp).

RESULTS

Efficient clustering of MAM-reactive T cells by antigen presented on dendritic cells. It is known that dendritic cells bearing mycobacterial antigen or alloantigen cluster antigen-specific T cells from bulk T-cell populations (17, 34, 35). These clustered T cells can be cloned and grown long term without loss of antigen reactivity. We determined whether partially enriched sources of dendritic cells cultured with MAM could similarly cluster SAg-reactive T cells. Bulk cultures formed clusters that were clearly visualized after 24 h of culture (Fig. 1A). These could be separated into discrete cluster and noncluster fractions (Fig. 1B and C) after 48 h as described previously (34, 35). After an additional 2 to 3 days of culture, blasts were released from the cluster but not the noncluster fractions (Fig. 1B).

When tested for SAg reactivity, T cells from the cluster fraction responded strongly to secondary stimulation with MAM compared with T cells from the noncluster fraction (Fig. 2), which were poorly responsive. Bulk (unseparated) T cells also proliferated to MAM in the presence of APCs, but the response was more modest than with the cluster-derived T cells. The addition of autologous APCs alone to cluster-derived T cells induced a significant response. We believe this is because clusters also contain SAg-bearing dendritic cells that remain bound to T cells. Analysis of T-cell clusters generated during an allo-MLR has shown that one of every three or four cells may consist of a dendritic cell (34). The presence of additional feeder cells with MAM-reactive T cells may potentiate the growth of already responding T cells. The addition of MAM as well would permit further expansion of activated T cells.

Dendritic cell–T-cell clusters are enriched for Vβ 17$^+$ T cells. It was previously shown that MAM preferentially

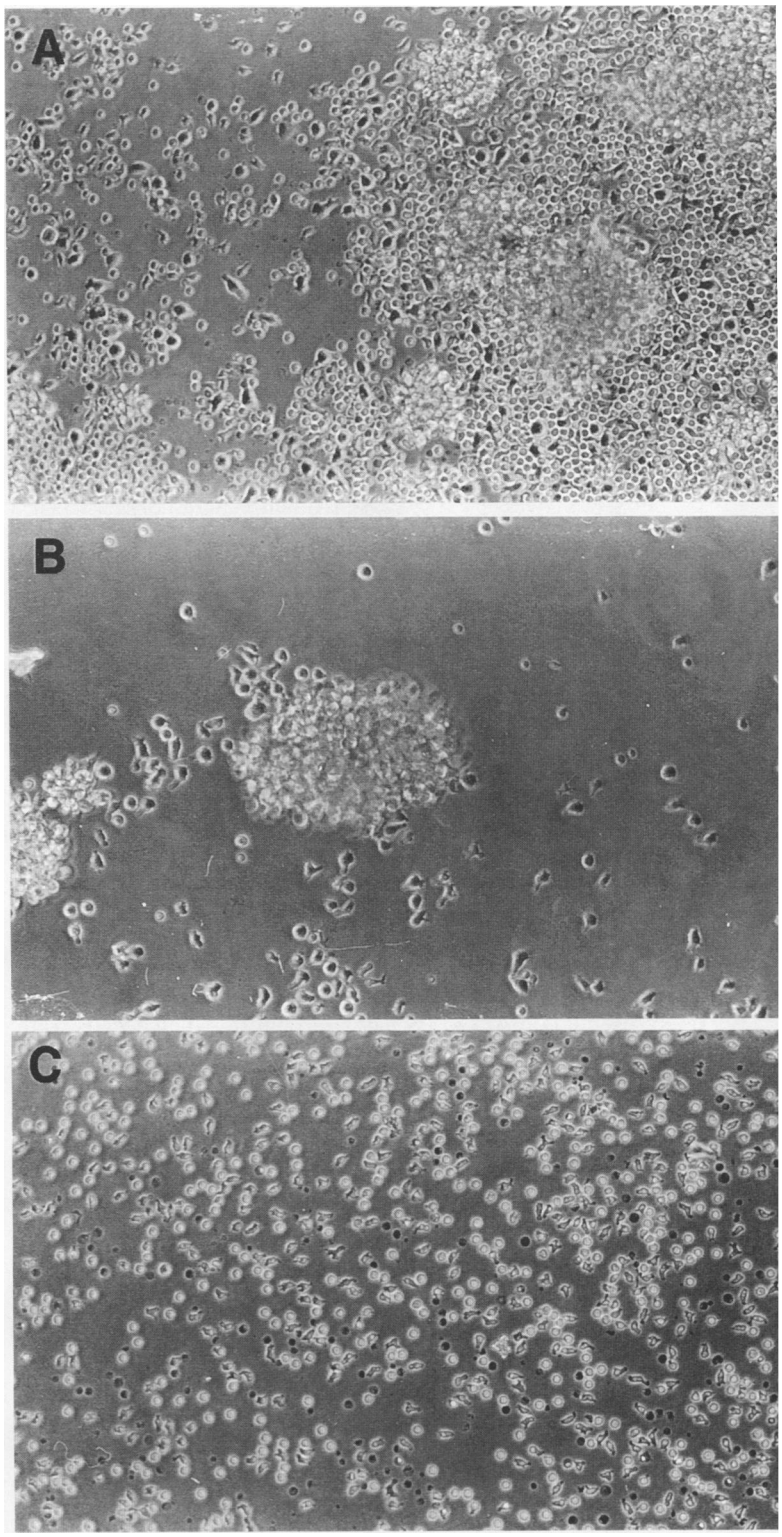

FIG. 1. T cells form clusters in the presence of MAM and dendritic cells. (A) T cells cultured for 2 days with MAM and partially purified dendritic cells. Note the clusters that form in the cultures. (B) T-cell clusters isolated from cultures in panel A by 1 × *g* sedimentation over 30% serum gradients. Note the blasts being released from the clusters. (C) Nonclustered T cells from panel A which lack clusters and large blasts. Magnification, 250×.

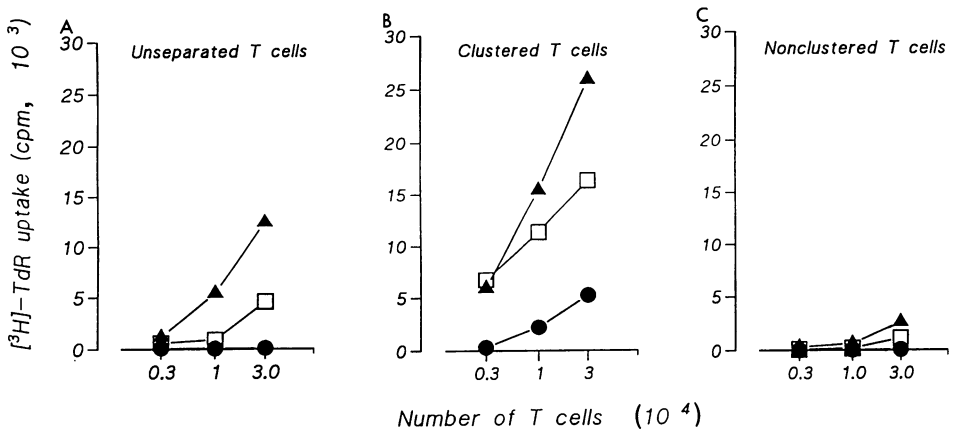

FIG. 2. MAM-reactive T cells are clustered by dendritic cells. Monocyte-depleted populations were cultured with MAM, on a continuous basis, and purified T cells from a normal donor. Forty-eight hours later, aggregates of T cells and APCs are evident (Fig. 1). The clusters were separated from nonclustered T cells over serum gradients, returned to culture for an additional 3 days, and then restimulated with MAM ± 10^5 APCs (syngeneic ER^- cells). (A) Bulk cultures; (B) clusters of T cells and dendritic cells; (C) Nonclustered T cells. ●, T cells alone; □, T cells plus APCs; ▲, T cells with APCs plus MAM, added at a final dilution of 1:4,000.

induces the expansion of Vβ 17^+ T cells in culture (19). To determine whether Vβ 17^+ T cells preferentially aggregated in the cluster-derived fractions, we stained bulk, clustered, and nonclustered T cells with a panel of MAbs directed towards specific TCR gene products. The T-cell populations were stained at two times, day 3 (Fig. 3A) and day 5 (Fig. 3B) of culture, corresponding to 1 and 3 days following the separation of the cluster fractions from bulk cultures over serum gradients. On day 3, there was a decrease in the percentage of $CD3^+$ and $CD4^+$ T cells in the cluster fraction, 38 and 50%, respectively, compared with those in the bulk and non-cluster-derived T cells (Fig. 3A). No enrichment of Vβ 17^+ T cells or other specific TCR^+ cells were seen, however. These findings suggested that the TCR complex was undergoing modulation following interaction with MAM presented on APCs. By day 5 of culture, when T cells were undergoing active proliferation, the percentage of CD3 and CD4 T cells in the cluster population was similar to that in the bulk cultures (70 to 73%) but remained significantly less than the numbers in unstimulated T-cell or nonclustered populations (85 to 89% [Fig. 3B]). The levels of $CD8^+$ cells remained unchanged.

The most striking change seen after staining with the panel of Vβ TCR MAbs was the enrichment of Vβ 17^+ cells in the cluster population (44%), corresponding to 60% of the CD3 cells (Fig. 3B). Although there is a relatively close correlation between the increase in the percentage of detectable Vβ 17^+ cells (2 to 44%) versus $CD3^+$ cells (38 to 73%) over time, it is likely that other responding T cells are involved in the MAM response, as we show below. The bulk population also had a relative enrichment of Vβ 17^+ cells (19%) compared with unstimulated (7%) and nonclustered (5%) T cells. The percentages of Vβ 8^+, 12^+, $5.2/5.3^+$, and $6.7a^+$ cells were similar in all the fractions studied. Thus, T-cell–dendritic cell clusters are a highly enriched source of Vβ 17 cells, presumably the MAM-reactive cells (Fig. 2), and these cells preferentially expand in the aggregates.

MAM-reactive clusters are an enriched source of lymphocytes that can be cloned with APC and IL-2. In earlier studies it was established that clusters containing antigen-reactive cells (e.g., to mycobacteria or alloantigens) could be efficiently expanded when cultured with APC and IL-2 (34, 35). MHC-mismatched APCs treated with mitogen (sodium periodate) suffice as feeder cells. These periodate-treated cells are highly efficient at potentiating the clonal growth of T cells, more so than autologous APCs and antigen (35). T-cell clones and lines that are expanded by using this system retain long-term reactivity to the antigen to which they were originally selected (35). The MAM reactivity of the cluster- versus non-cluster-derived T cells was evaluated by developing long-term cell lines in the presence of allogeneic sodium periodate-treated APCs. T cells from both groups were plated at 1 or 10 cells per well in 96-well microtiter plates, together with APCs and IL-2. Cloned lines were usually visualized as early as day 7 to 10 after plating. It was possible to test 83 T-cell lines from 108 visualized in the cluster group and 20 from an initial total of 30 lines from the noncluster group. Of cloned lines obtained from clustered T cells, 76% (63 of 83) were MAM reactive (SI, >10), while no reactivity was seen in non-cluster-derived lines (0 of 20 [Fig. 4]). Of MAM-reactive lines, 49% (31 of 63) expressed Vβ 17 on some or all cells as determined by staining with C1 MAb (Fig. 5), confirming Vβ 17 as the major TCR gene product used by MAM-reactive T cells. Only one non-cluster fraction-derived line stained with C1, but this line failed to proliferate to MAM. All Vβ 17^+ T-cell lines from the cluster fraction proliferated to MAM, except for two (65 and 77). The latter was responsive to MAM in one of three experiments, however.

Several lines were also tested for the expression of other Vβ gene products (Fig. 5). Three cluster-derived cell lines (26, 32, and 44) had significant numbers of Vβ 12^+ cells (25 to 80%), and all three proliferated to MAM. One cell line (89) from the noncluster fraction had significant numbers of Vβ 12^+ T cells (>70%); however, it did not proliferate to MAM.

In a similar fashion, three cluster-derived cell lines (60, 59, and 10) contained high percentages of Vβ 8-expressing T cells (as determined by using the MAb Ti3a) and were MAM reactive, while another non-cluster-derived line (87), also containing $Ti3a^+$ cells, was consistently MAM nonreactive (Table 2). Two cluster-derived cell lines expressed Vβ 5.1 (as determined by using the MAb LC4) and were MAM reactive.

In summary, dendritic cell–T-cell clusters are efficient

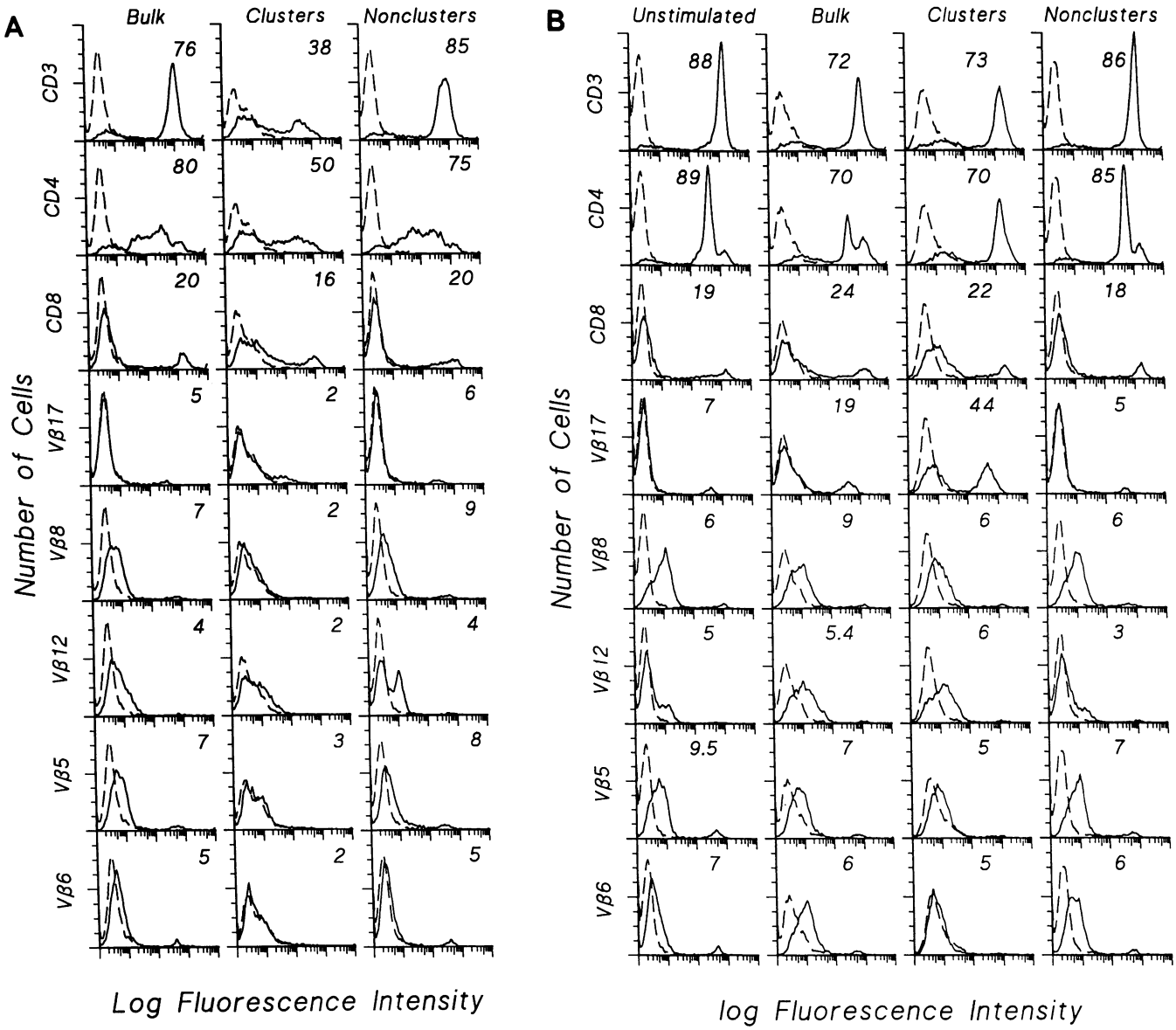

FIG. 3. Phenotype of human blood T cells after stimulation with MAM presented on dendritic cells. Purified T cells were cultured for 2 days with MAM and partially enriched populations of dendritic cells. They were then separated into cluster and noncluster fractions over serum gradients and cultured for another day (A; day 3) or an additional 3 days (B; day 5). Control populations consisted of unstimulated or unseparated T cells (bulk cells). T cells were stained with a panel of MAbs to T-cell markers and Vβ gene products. Note the enrichment of Vβ 17^+ cells in the cluster fraction at day 5. The indicated percentage in each panel is not corrected for the total amount of $CD3^+$ TCR^+ T cells in each culture.

units for the enrichment of MAM-reactive T cells that can be cloned. The cell lines thus generated contain a majority of Vβ 17^+ cells, most of which are MAM reactive. In addition, there is selection of MAM-reactive T cells that express other Vβ gene products. Lines derived from the nonclustered fraction occasionally can express the MAM-reactive Vβs, but they do not respond to MAM.

Correlation of MAM reactivity and Vβ usage determined by MAb staining and by PCR. Several T-cell lines were evaluated further for long-term retention of MAM reactivity and for Vβ expression by using immunofluorescence (Table 2) and an RT-based PCR (Fig. 6). Several conclusions can be drawn from the data. First, some lines appear to be clonal in that 100% of the cells react with the same Vβ-specific MAb and a single band is seen by PCR analysis (e.g., lines 1, 74, 77, 79, and 27). Other lines are definitely not clonal as judged by the same criteria (e.g., lines 10, 20, 45, and 2). Second, PCR analysis allowed other Vβ gene products expressed by MAM-reactive T-cell lines to be identified. Two apparently clonal lines (3 and 19) expressed Vβ 7, for instance. There is no Vβ 7-specific MAb, demonstrating the utility of PCR analysis to detect a more complete array of Vβ genes used by MAM-reactive T cells. Thus, Vβ 20 (lines 34 and 20) and Vβ 12 (lines 26, 32, and 44 [Table 2; Fig. 5]) are also used by MAM-reactive T cells. We could not confirm that Vβ 10 (detected in lines 10 and 20 [Table 2]) is used by MAM-

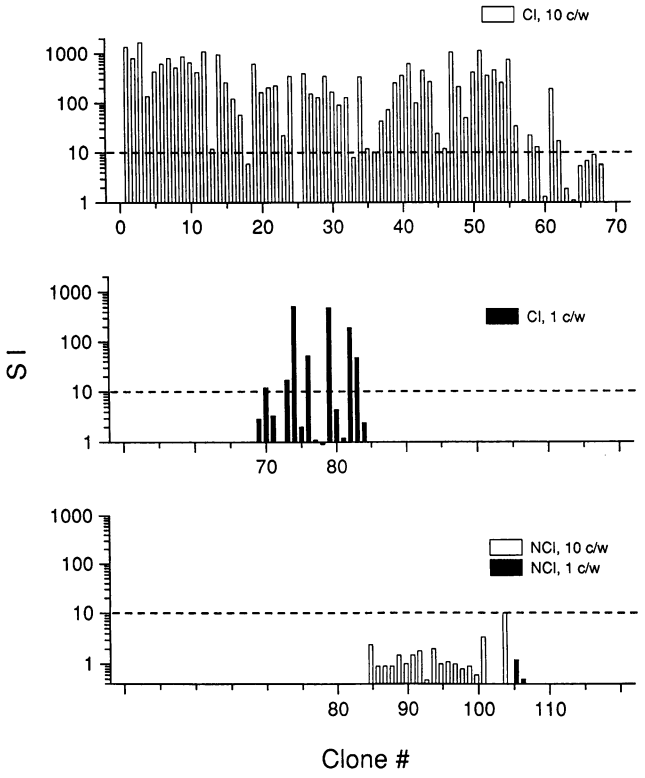

FIG. 4. MAM reactivity resides in cluster- but not non-cluster-derived T-cell clones. T cells derived from the cluster and noncluster fractions were cloned by limiting dilution at 1 or 10 cells per well in microtiter plates, with IL-2- and sodium periodate-modified allogeneic feeder cells. After 14 days, the reactivities of the clones were tested in the presence or absence of MAM plus syngeneic APCs. Results are expressed as SIs, where SI = [^{3}H]TdR incorporation (counts per minute) of cells plus MAM and APCs/[^{3}H]TdR incorporation (counts per minute) of cells plus APCs alone. An SI of 10 (dashed line) was used to distinguish between MAM-reactive versus nonreactive T-cell clones. A total of 63 of 83 cluster-derived lines are MAM reactive compared with 0 of 20 non-cluster-derived lines.

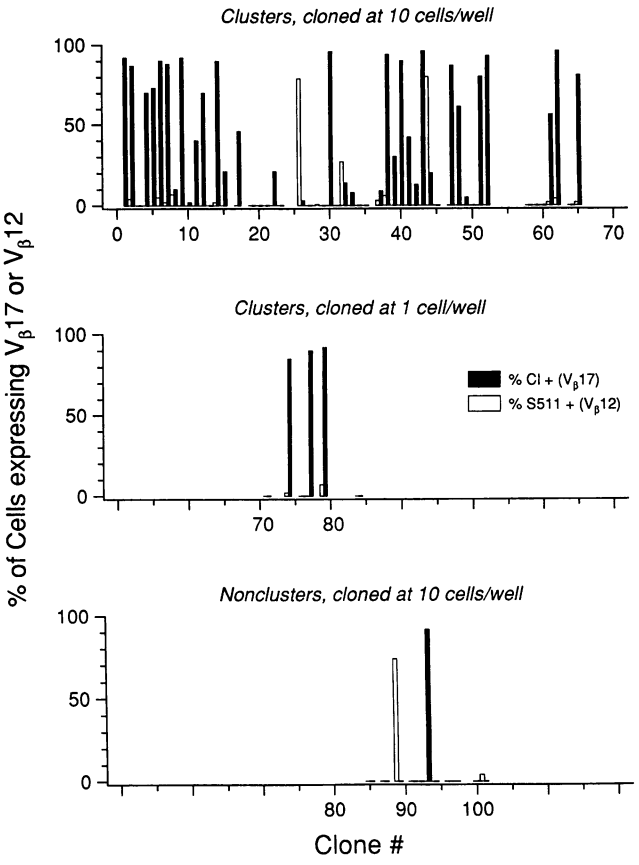

FIG. 5. Phenotype of cluster- and non-cluster-derived T-cell clones and lines. T cells cloned by limiting dilution, as described in the legend to Fig. 4, were stained with anti-Vβ 17 (Cl) or Vβ 12 (S511) MAbs and then with phycoerythrin-conjugated goat anti-mouse immunoglobulin. Results are expressed as a percentage of cells expressing Vβ 17 or Vβ 12. A total of 31 of 63 (49%) of cluster-derived lines contained significant numbers of Vβ 17$^+$ cells compared with only 1 of 11 non-cluster-derived lines. A total of 3 of 63 cluster-derived lines and 1 of 11 non-cluster-derived lines contained Vβ 12$^+$ cells.

reactive T cells, since it was only seen in lines that expressed other MAM-reactive Vβ gene products. In addition, in several lines (lines 28, 31, and 50) with clear MAM reactivity, no Vβ expression could be detected with the panel of PCR primers used, suggesting that yet other Vβs may be expressed by MAM-reactive cells. Third, the complete correlation between Cl reactivity and Vβ 17 mRNA expression detected by PCR demonstrates the specificity of this MAb. Fourth, several cell lines retained MAM reactivity when tested repetitively over several months, including those expressing Vβ 17, 8, 7, and 5.1. The response also occurred in the presence of allogenic APCs, indicating that the lines are responding to MAM and are not class II restricted. A total of 17 cell lines were tested with APCs from seven different donors, and all demonstrated MAM responsiveness, without evidence of alloreactivity. Repeated analysis for MAM reactivity was not always concordant with a given cell line, however. This could be due to shifts in the composition of cell lines as seen when PCR analysis was repeated at a later time (e.g., lines 10 and 20). Alternatively, some cell lines may have become anergic with prolonged culture.

DISCUSSION

MAM as a model SAg in RA. The SAg produced by the pathogen *M. arthritidis* (MAM) or similar agents are of special interest in the pathogenesis of RA. The speculated role of MAM-like agents in this chronic arthritis is based on two findings: (i) injection of *M. arthritidis* organisms into susceptible strains of mice leads to a chronic polyarthritis (13, 14), and (ii) the ability of MAM to expand T cells expressing Vβ 17 (19), a TCR phenotype that is expressed by oligoclonal populations in RA synovial effusions (23). The description of preferential skewing of TCR products in synovial cells (23, 33), however, does not prove a direct role for these SAgs, or MAM-like molecules, in disease pathogenesis. Furthermore, *M. arthritidis* has not been definitely isolated from synovial tissue and is not known to be a human pathogen. Nevertheless, it is possible that a related SAg with similar specificities is involved in RA. Evidence to support a role for SAgs in autoimmune arthritis is the recent finding that MAM can trigger or exacerbate murine collagen-induced arthritis, apparently in a Vβ-restricted manner (12).

More direct approaches toward detecting SAgs on APCs

TABLE 2. MAM reactivity and Vβ profile of T-cell lines[a]

Clone	MAM reactivity[b]	MAb staining[c] (%)	Vβ gene(s)[d] (by PCR)
Cluster derived			
1	+2/2	C1, 100	Vβ 17
74	+5/5	C1, 100	Vβ 17
77	+1/3	C1, 100	Vβ 17
79	+5/6	C1, 100	Vβ 17
2	+2/2	C1, 85	Vβ 17, 7
8	+1/1	C1, 15	Vβ 17
65	−1/1	C1, 81	ND
45	+1/2	C1, <5	Vβ 17, 8, 13.2
60	+1/1	Ti3a, 54; C37, 28	ND; ND
59	+1/1	Ti3a, 90	Vβ 8
10	+1/1	Ti3a, 80, 88	Vβ 8, 10 (Vβ 6, 8, 10)
3	+5/6	−	Vβ 7
19	+3/3	−	Vβ 7
76	+3/6	LC4, 80	Vβ 5.1
27	+4/4	LC4, 100	Vβ 5.1
34	+5/5	−	Vβ 20
20	+1/1	OT145, 12	Vβ 6, 7, 10, 17, 20 (Vβ 2, 10, 17, 20)
21	+1/1	OT145, 8	ND
26	+3/3	S511, 90	Vβ 12
28	+2/2	−	No band
31	+2/2	−	No band
50	+1/1	−	No band
58	+1/1	−	ND
49	+1/1	−	ND
7	+3/3	−	ND
71	+3/6	−	ND
83	+2/2	−	ND
Non-cluster derived			
89	−2/2	S511, 80, 90	Vβ 12, 2
92	−2/2	−	Vβ 7
94	−2/2	−	Vβ 19
96	−4/4	S511, 10	Vβ 13.2
100	−2/2	−	Vβ 18
93	−2/2	C1, 100	ND
87	−3/3	Ti3a, 22	ND
84	−1/1		ND
85	−2/2		ND
91	−2/2		ND
97	−2/2		ND
101	−2/2		ND

[a] Several T-cell lines derived from cluster and noncluster fractions were analyzed for MAM reactivity and Vβ expression by using a panel of MAbs and/or PCR. Note that in addition to expression of Vβ 17, MAM-reactive lines also express Vβ 7, 5.1, 20, 8, 12, and possibly 10.

[b] MAM reactivity refers to the MAM responsiveness of individual T-cell lines or clones, where + represents a SI of >10 and − represents a SI of <10, as described in Materials and Methods. In addition, the frequency of positive or negative responses of each line to MAM over a several-month period is listed.

[c] MAb staining refers to the percentage of T cells in individual lines that stained with a panel of MAbs recognizing specific Vβ TCR components, which are described in Materials and Methods. −, no cells stained with the panel.

[d] ND, not determined; no band, no Vβ expression could be detected with the panel of PCR primers used.

from synovial tissue are required to pursue this hypothesis. Human pathogens with MAM-like SAg activity, which in very small amounts could trigger T cells, may exist (7, 20) (analogous to the Mls antigens [22, 31]). Therefore, it is necessary to devise sensitive strategies to detect these proteins on pertinent APCs. Direct immunoprecipitation of SAgs with anti-MHC class II MAbs, e.g., from cells expressing Mls antigens, is technically difficult (43). Consequently, a panel of sensitive, well-characterized MAM-reactive clones or lines would be useful probes to detect MAM or related molecules in diseases like RA, particularly on APCs derived from the affected tissue.

Dendritic cells as a means to immunoselect MAM-reactive T cells. To generate enriched populations of MAM-reactive T cells, we employed several special properties of dendritic cells as immunoadsorbents to select out antigen-reactive cells. First, several prior studies have established that dendritic cells can function as reservoirs of antigen and can induce antigen-specific immunity even when injected into whole animals (24–26). In vitro, dendritic cells expressing

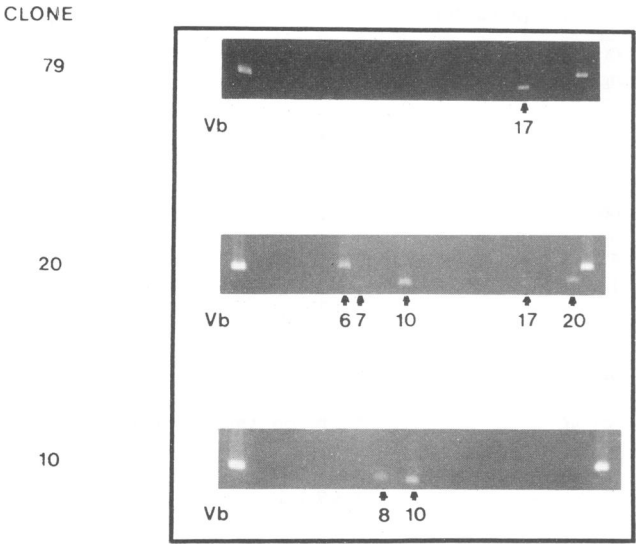

FIG. 6. Vβ analysis by PCR. Examples of Vβ analysis by PCR (for cell lines 79, 20, and 10). The lateral lanes for each gel contain control PCRs (Vβ 6 and Cβ) from a plasmid substrate (326 bp). Vβ1 to Vβ20 were loaded from left to right. Positive Vβs are indicated. A band in the Vβ 15 lane (clone 20) represents too large a fragment and is therefore artefactual.

antigen (e.g., allogeneic antigens and mycobacterial antigens) sequester antigen-reactive T cells into clusters which can subsequently be cloned by limiting dilution (34, 35). This feature is a well-defined property of dendritic cells, since other APCs (B cells and monocytes) do not enter the clusters (17, 34). At the clonal level, antigen-reactive cells can be expanded and maintained long term in the apparent absence of additional antigen. If bulk T cells are used as the initial population for generating antigen-specific clones, a much lower incidence of antigen-specific T-cell lines is obtained (35). Second, dendritic cells are potent APCs for SAg presentation, especially MAM (4). Third, SAgs (SEA and SEB) can be applied as a measurable pulse onto dendritic cells, and T-cell proliferation is efficiently induced. This occurs even after after pulses of picomolar or femtomolar amounts of SAg (4, 7).

When bulk T cells were cocultured with partially enriched dendritic cells and MAM, large clusters were visualized (Fig. 1), analogous to other systems (17, 34, 35). When compared with the bulk T cells and the noncluster fraction, the clusters were enriched in MAM-reactive T cells. We also found that a major population of cells undergoing expansion in this fraction were Vβ 17^+ (44% [Fig. 3B]), initially assessed by staining with the MAb C1 on day 5 of the culture. This MAb was generated to a MAM-reactive T-cell line and recognizes a majority of the T cells in the original immunogen (19). The clustered population had significantly larger numbers of Vβ 17^+ cells than the MAM-stimulated bulk T-cell (19%) or nonclustered T-cell (5%) groups. No enrichment of several other Vβ profiles was observed, confirming that Vβ 17 is the major TCR used by MAM-reactive cells.

To detect the increase in Vβ 17^+ cells, it was necessary to stain the T-cell populations at day 5 of culture. When examined at an earlier time, day 3 (Fig. 3A), the clustered cells expressed few Vβ 17^+ cells but were notably reduced in numbers of $CD3^+$ and $CD4^+$ cells. These results are most consistent with a SAg modulation of the TCR-CD3 complex, since by day 5 CD3 is reexpressed and approaches the level seen in unstimulated populations.

Establishment and characterization of MAM-reactive T-cell lines and clones. Once selected, MAM-reactive cells could be cloned by limiting dilution and maintained long term with periodate-treated feeder cells, as previously described (16, 34). The most striking finding was the efficiency with which antigen-specific cell lines developed from the cluster fraction. Of all starting lines, 76% expressed MAM reactivity compared with 0% of lines obtained from the noncluster fraction. Limiting dilution did not appear to affect the composition of the MAM-reactive T-cell population, since the fraction of cell lines expressing Vβ 17 (49% [Fig. 4]) was similar to the number of cells that were Vβ 17^+ in the original cluster fractions (44% [Fig. 3B]). Thus, our data suggest that most or all MAM-reactive cells can be sequestered into the cluster units.

In conjunction with PCR analysis, the cluster approach for cloning antigen-specific lines permitted the identification of other MAM-reactive Vβ TCR components. For example, Vβ 5.1, 7, 8, 12, and 20 all seem to be MAM-responsive components of the TCR. We did not detect Vβ 3.1, 11.1, or 13.1, as described elsewhere (3). However, differences in the responding T cells may relate to polymorphisms of the MHC and TCR genes in different donors (11, 21, 36). A similar evaluation of MAM-reactive lines with different donors would be required to address this issue. We also show here the complete concordancy of the C1 MAb in detecting Vβ 17 with the PCR results.

Many of our lines retained MAM reactivity over several months and were not class II restricted, indicating that they were responding to the SAg property of MAM.

Advantages of using dendritic cells as immunoadsorbents to select SAg-reactive T cells. This new approach to select, enrich, and clone for SAg-responsive T cells has several advantages over standard procedures to generate SAg-specific cell lines. Besides its relative simplicity, speed, and efficiency (16, 34), it relies on the use of APCs (dendritic cells) that are known to present SAgs and other antigens in situ (26, 27). For example, dendritic cells can present Mls antigens to thymocytes in vivo, leading to anergy of the responding T cells (27). Since the responding T cells are directly cloned from the clusters and the clusters contain all the SAg-reactive T cells, we avoid the pitfalls of expanding bulk populations of T cells, as would occur in most routine cloning protocols. In those, T cells are expanded with IL-2 following the addition of antigen, and IL-2-responsive but non-antigen-reactive cells may also undergo proliferation. Furthermore, T cells do not expand as efficiently as in our system, in which we add an excess of feeder cells in the form of periodate (mitogen)-treated APCs after the selective binding step.

This approach also allows the identification of several minor but relevant SAg-reactive Vβs, and in preliminary studies, we have shown its applicability for the study of Vβs used by other SAgs (SEE and SEB; data not shown).

Cloning of the noncluster T-cell populations was useful, since it permitted the identification of Vβ 17^+ and 12^+ cells that are not MAM responsive (Fig. 4 and 5, lines 93 and 89). Consequently, by sequencing the α and β chains of the TCR from such cell lines, it may be possible to identify residues critical for MAM binding. Thus, the panel of lines will also be useful for analyzing the contribution of TCR components other than Vβ to MAM reactivity.

Detection of MAM-like SAgs in autoimmune disease. The applicability of these cell lines to test for the presence of

MAM or MAM-like SAgs in disease states is notable for several reasons. Many of these lines respond to very low concentrations of MAM, on the order of 1 pM (data not shown). Thus, small amounts of SAg on APCs may be detectable by using these cell lines. We have shown this to be the case for dendritic cells pulsed with very low amounts (5 pM) of SEA where there is occupancy of only 2,000 MHC class II molecules per cell, a number sufficient to induce the proliferation of bulk T-cell populations (7). It is also evident that dendritic cells can hold onto SEA in an immunologically intact form for at least 2 days (7) and Mls antigens over several days (27). Dendritic cells are such potent APCs for SEA, that following a pulse with low amounts of SEA (5 pM), they are functional in T-cell assays even at 1 cell per 1,000 T cells. Rheumatoid effusions contain relatively large numbers of dendritic cells (5, 6, 46), and these cells are capable of presenting SAgs (data not shown). Given their potency, we speculate that MAM-like SAgs could be detected by using few cells when added to sensitive MAM lines. Since it is not clear whether SAg will play a role in disease initiation or maintenance of the autoimmune response, APCs from different stages of disease would need to be assessed. We are currently addressing these possibilities.

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