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Presentation of T-Cell Epitopes Assembled as Multiple-Antigen Peptides to Murine and Human T Lymphocytes

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Multiple-antigen peptide (MAP) constructs containing different T- and B-cell epitopes were assessed for their ability to be specifically recognized by murine and human T-cell clones. The different synthetic MAP constructs consisted of a malaria T-cell epitope or of a human universal tetanus toxin helper T-cell epitope colinearly synthesized with B-cell epitopes from the circumsporozoite proteins of different malaria parasites. All constructs were able to stimulate specifically T-cell clones. Interestingly, T-cell epitopes assembled as MAP constructs did not require processing for the specific stimulation of murine and human T-cell clones, as shown by retention of their stimulatory effect in the presence of glutaraldehyde-fixed antigen-presenting cells. However, processing was required for most of the synthetic constructs containing both T- and B-cell epitopes. Thus, the requirement for processing of these constructs seems to be dictated by the nature of the B-cell epitope present.

Several lines of research are being pursued towards the development of malaria vaccines. One strategy consists of the design of subunit constructs containing defined B- and T-cell epitopes obtained by genetic engineering or by chemical synthesis (reviewed in reference 10). One target of this research is represented by the first developmental stage of the plasmodial parasite, the sporozoite, which is inoculated by infected anopheline mosquitoes into the host. The surface of the sporozoite is covered by a major antigen, the circumsporozoite (CS) protein, which contains a series of tandemly repeated amino acids representing the immunodominant B-cell epitope. In some experimental models, anti-repetitive peptide antibodies have been able to confer immune protection against infection (6, 16). One of the approaches taken towards improving the immunogenicity of synthetic malaria constructs involves assembling them as multiple-antigen peptides (MAP). These constructs are made of a core of lysines, the α and ε amino groups of which are used as growing points for the peptide chains (12). MAP containing synthetic epitopes from foot-and-mouth disease virus (4), *Schistosoma mansoni* (15), and different species of plasmodia (1, 3, 7, 9, 13, 14) have been produced. In the present study, we examined whether the assembling of T-cell epitopes as MAP could affect their processing by antigen-presenting cells (APC) and their recognition by specific T cells.

We had previously shown (5) that the 21-mer amino acid sequence from residues 59 to 79 (YNRNIVNRLLGDAL NGKPEEK) of the *Plasmodium yoelii* CS protein (referred to as Py1) contained T-cell epitopes recognized by *H-2*^{d} and *H-2*^{b} mice and subsequently mapped within the 13-mer sequence from residues 59 to 71 (YNRNIVNRLLGDA; referred to as Py1T) (2). The repetitive (QGPGAP) sequence of the *P. yoelii* CS protein (referred to as Py3 or Py4) (Fig. 1) was only immunogenic in *H-2*^{b} strains of mice (5). A series of linear or branched peptides were produced (14) to contain either the Py1T epitope alone or the Py1T epitope plus the repetitive Py3 epitope (Fig. 1). These constructs were used to immunize 8- to 12-week-old BALB/c and C57BL/6 mice at the base of the tail (50 μg in complete Freund's adjuvant [Difco Laboratories, Detroit, Mich.]) on day 0 and 15 days later (in incomplete Freund's adjuvant [Difco]). Serum samples were tested for the presence of specific antibodies by an enzyme-linked immunosorbent assay (ELISA) (5). The proliferative responses of lymph node cells from immunized animals and of Py1-specific T-cell clones were measured as described in detail elsewhere (5).

In a first set of experiments, the Py1 epitope contained in the linear (Py1-Py3) and branched (MAP4-Py1-Py3) hybrid peptides was shown to behave as a helper T-cell epitope for the induction of anti-Py3 antibodies (Fig. 2A). In fact, BALB/c mice did not respond to immunization with the Py4 peptide alone, confirming data already published by us (5), but produced high titers of anti-Py4 immunoglobulin G antibodies after immunization with linear and branched hybrid peptides. As expected because of the *H-2*^{b} restriction of the response to the repetitive epitope, C57BL/6 mice always responded to immunization with either construct containing Py3. As shown in Fig. 2B, lymph node cells from C57BL/6 mice immunized twice with different constructs exhibited a significant proliferative response when restimulated in vitro by any of the four different constructs containing the Py1T epitope. Similar results were obtained with lymph node cells derived from immunized BALB/c mice (data not shown).

We had previously produced a large panel of Py1-specific CD4$^+$ T-cell clones from C57BL/6 and BALB/c mice immunized with the Py1 peptide (5). We investigated whether these clones would recognize the T-cell epitopes contained in the different constructs. Figure 3 shows the proliferative patterns of some BALB/c- and C57BL/6-derived clones in the presence of the four constructs tested. All these clones belonged to the Th1 subset of the CD4$^+$ cell population, as determined by their ability to produce gamma interferon but

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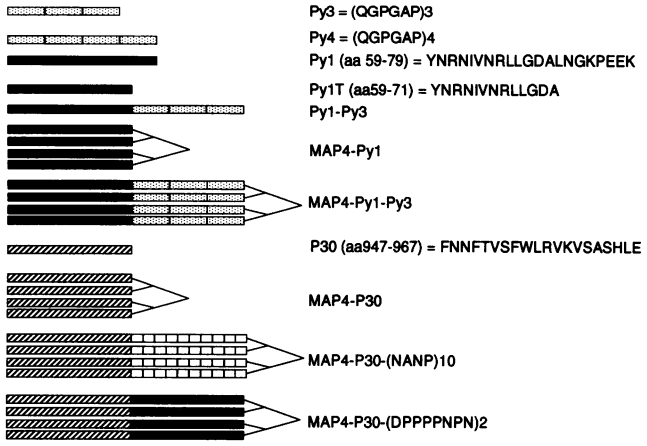

FIG. 1. Schematic representation of the different constructs used in the study. Py3 and Py4 represent three and four repeats, respectively, of the QGPGAP sequence of the *P. yoelii* CS protein. Py1 represents the *P. yoelii* CS protein sequence from residues 59 to 71. P30 represents the tetanus toxin sequence from residues 947 to 967. $(NANP)_{10}$ represents 10 repeats of the NANP sequence of the *P. falciparum* CS protein. $(DPPPPNPN)_2$ represents two repeats of the DPPPPNPN sequence of the *P. berghei* CS protein. MAP4 represents a MAP with a core of three lysines and four copies of peptides. Numbers in parentheses indicate the amino acid (aa) positions starting from the first methionine of the signal sequence.

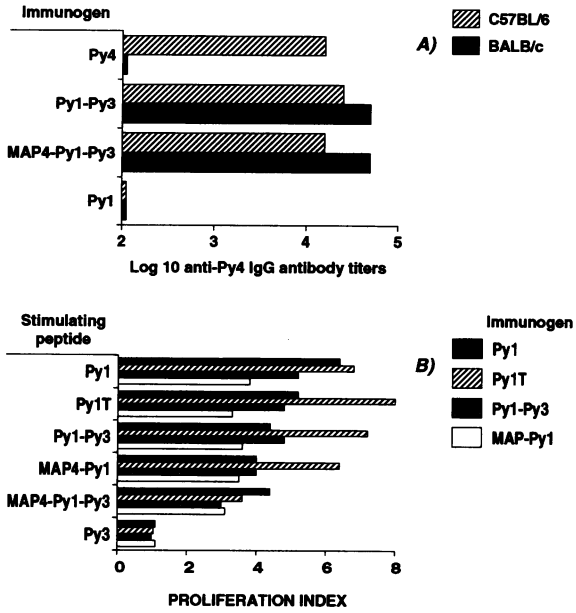

FIG. 2. Immunogenicity of different Py1-based peptide constructs. C57BL/6 and BALB/c mice were immunized twice with the different constructs (50 μg). (A) Seven days later, mice were bled and serum samples were tested by an ELISA with the Py4 peptide as the solid phase (1 μg/ml). (B) One week later, lymph node cells were removed and their proliferative responses in the presence of the different constructs were assayed. Resuspended lymph node cells were seeded in 200-μl cultures at 2×10^5 cells per culture in the presence of a peptide. Four days later, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 h later, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting. Results obtained with peptides at 30 μg/ml and with C57BL/6 lymph node cells are shown. Background values were 2,500 cpm.

not interleukin 5 [5]. Py1-specific $CD4^+$ T-cell clones were able to recognize the Py1T epitope within every construct. Furthermore, the pattern of proliferation for each clone was comparable in the presence of linear and branched constructs (Fig. 3). These results also clearly showed that the Py1T epitope was correctly reproduced in the hybrid MAP containing the repetitive Py3 sequence.

We next tested whether T-cell epitopes assembled as linear or branched MAP also containing B-cell epitopes needed to be processed by APC to be correctly presented to T-cell clones. $CD4^+$ T-cell clones were stimulated by the different Py1T-based synthetic constructs in the presence of syngeneic spleen cells as APC, fixed or not fixed with glutaraldehyde to prevent antigen processing (11). Figure 4 shows the results obtained with some BALB/c- and C57BL/6-derived T-cell clones. The results obtained show that Py1 (21 amino acids) and Py1T (13 amino acids) did not require processing to induce proliferation of the clones. Interestingly, the tetra-branched MAP4-Py1 construct did not require processing, since similar proliferative responses of the T-cell clones were obtained with either fixed or nonfixed APC. On the other hand, proliferation in the presence of both the linear hybrid Py1-Py3 and the tetra-branched hybrid MAP4-Py1-Py3 was abrogated when glutaraldehyde-fixed APC were used (Fig. 4), suggesting the requirement for their processing to induce proliferation. The same experiments were repeated in the absence of fetal calf serum for the first 24 h, thereby excluding a possible effect of serum proteases on the cleavage of the peptide constructs. Results the same as those shown in Fig. 4 were obtained (data not shown). These results ruled out the possibility that serum proteases cleaved the MAP4-Py1, Py1-Py3, and MAP4-Py1-Py3 constructs.

To determine whether similar results could be obtained with other MAP constructs and with human cells, we carried out experiments with MAP containing the universal T-cell epitope P30 from tetanus toxin (8) and containing or not containing *P. falciparum* CS protein (NANP) or *P. berghei* CS protein (DPPPPNPN) repeats (11). We evaluated the ability of human P30-specific T-cell clones (8, 14) to proliferate in the presence of constructs P30, MAP4-P30, MAP4-P30-$(NANP)_{10}$, and MAP4-P30-$(DPPPPNPN)_2$ (Fig. 1). The proliferation assays were performed as previously described (8, 14) with irradiated Epstein-Barr virus-transformed B (EBV-B) lymphocytes. The results obtained with the DR5-restricted KT30 clone (14) are shown in Fig. 5. P30-specific T-cell clones recognized MAP4-P30 equally well in the presence of fixed and nonfixed APC. For the MAP4-P30-$(NANP)_{10}$ construct, no significant proliferation was observed in the presence of fixed APC, suggesting that processing of this construct by APC was required for proliferation, similar to the results obtained with the MAP-Py1-Py3 construct. However, processing did not appear to be required when P30-specific human T-cell clones were stimulated with the MAP4-P30-$(DPPPPNPN)_2$ construct (Fig. 5).

Several conclusions can be drawn from these results. First, the genetic restriction of Py3 could be overcome in *H-2^d* mice with the hybrid linear or branched construct containing both the Py1T and the Py3 epitopes. This result clearly shows that the Py1 peptide is a helper T-cell epitope. Second, the hybrid branched MAP4-Py1-Py3 construct was not more immunogenic than the linear form at both anti-peptide antibody and proliferative levels. Experiments are in progress to determine whether the anti-sporozoite antibody response is improved after immunization with these hybrid

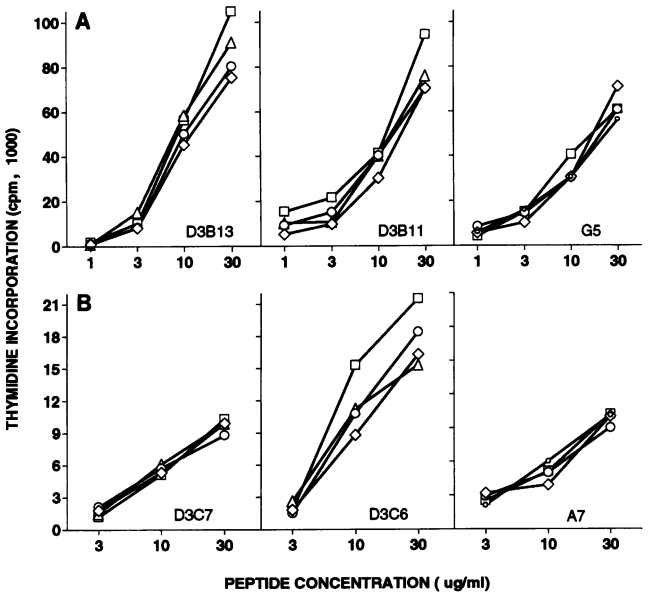

FIG. 3. Py1-specific CD4+ T-cell clones recognize the Py1 sequence in the context of linear and branched hybrid MAP. Py1-specific clones were derived from C57BL/6 and BALB/c mice immunized twice with Py1 in complete Freund's adjuvant as described previously [5]. These clones were highly specific for the Py1 sequence (2, 5). D3B13, D3B11, and G5, three BALB/c-derived T-cell clones, and D3C7, D3C6, and A7, three C57BL/6-derived T-cell clones, were stimulated in vitro with different concentrations of Py1-based constructs in the presence of syngenic irradiated spleen cells as APC. The proliferation assays were performed as described in the legend to Fig. 2. Each point represents the mean for triplicate wells (standard deviations never exceeded 15% of the mean). Background values did not exceed 1,000 cpm. Symbols: □, Py1; △, Py1T; ○, Py1-Py3; ◇, MAP4-Py1-Py3; ○, MAP4-Py1.

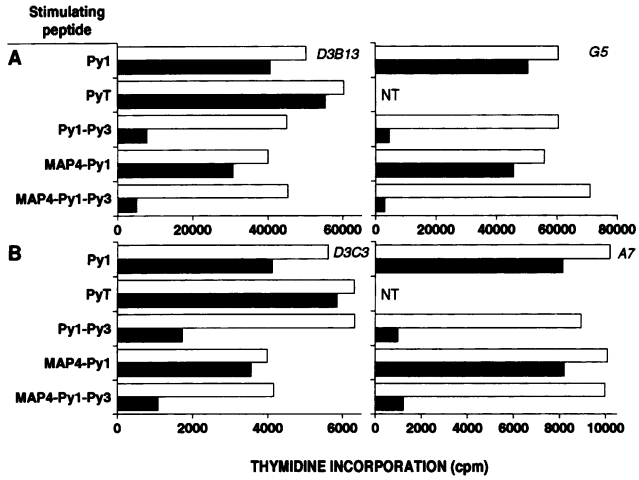

FIG. 4. Py1 peptide does not require processing when it is assembled as a MAP but does when it is assembled as a linear or branched peptide containing the Py3 sequence. D3B13 and G5, two BALB/c-derived T-cell clones, and D3C3 and A7, two C57BL/6-derived T-cell clones, were stimulated in the presence of different Py1-based constructs (results with 30 μg/ml are shown) and of APC fixed (closed bars) or not fixed (open bars) with 0.025% glutaraldehyde. The proliferation assays were performed as described in the legend to Fig. 2. In the experiment shown in this figure, background values were 1,500 cpm for BALB/c clones and 500 cpm for C57BL/6 clones. Similar results were obtained in other experiments with the same and other Py1-specific T-cell clones. Each bar represents the mean for triplicate wells (standard deviations never exceeded 15% of the mean). NT, not tested.

MAP constructs. One could hypothesize that MAP may increase the immunogenicity of peptides poorly immunogenic per se (13) and may have less of an effect on strong epitopes, such as Py1. Third, the recognition of the Py1 epitope by CD4+ T-cell clones requires previous processing by APC of the linear and branched hybrid constructs containing the Py3 sequence; however, this is not the case for MAP4-Py1. Little is known about the processing of the MAP structure and the cleavage of α and ε amide linkages. Our studies of the processing of these constructs suggest that the Py1 and P30 T-cell epitopes retain their ability to bind major histocompatibility complex class II molecules even in their macromolecular form (MAP4-Py1 and MAP4-P30). However, processing was required when cells were challenged in vitro with linear or branched Py1T constructs also containing the Py3 sequence. The elongation of the Py1 peptide by the addition of the Py3 sequence may alter the conformation and/or the affinity of the construct for major histocompatibility complex class II molecules, rendering necessary the processing of the hybrid peptide in its linear or branched form. An identical conclusion could be drawn for human T cells stimulated by the MAP construct containing P30 and the NANP sequence. However, this conclusion did not seem to apply to all MAP constructs, since MAP4-P30-(DPPPP NPN)$_2$ did not need processing to stimulate human T-cell clones. This result could have been due to the characteristics of the amino acids contained in the B-cell epitope, to the length of peptides reproducing B-cell epitopes, and/or to the structure of hybrid peptides assembled as MAP. The use of EBV-B cells as APC did not appear to have a major influence on the requirement for processing, since MAP4-P30-(NANP)$_{10}$ and MAP4-P30-(DPPPPNPN)$_2$ exhibited different proliferative patterns in the presence of irradiated EBV-B cells.

From these experiments, we can conclude that, at least in these present models, MAP assembling of T-cell epitopes

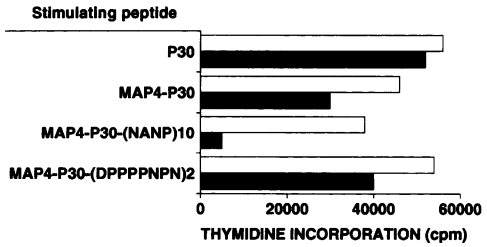

FIG. 5. Recognition of MAP constructs by human T-cell clones. The DR5-restricted KT30 human T-cell clone (8, 14) was stimulated in the presence of different P30-based constructs (1 μg/ml) and of APC fixed (closed bars) or not fixed (open bars) with 0.025% glutaraldehyde. KT30 cells were seeded in 200-μl cultures at 10^4 cells per well in the presence of irradiated (6,000 rads) syngeneic EBV-B cells (10^4 cells per well) and a peptide. Two days later, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 18 to 24 h later, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting. In the experiment shown in this figure, background values were 1,000 cpm. Similar results were obtained in other experiments with the same and other P30-specific human T-cell clones. Each bar represents the mean for triplicate wells (standard deviations never exceeded 12% of the mean).

does not represent per se a prerequisite for processing, which instead appears to be dictated by the presence of an additional sequence [e.g., Py3 or $(NANP)_{10}$] in linear as well as in branched constructs.

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