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Induction of Protective Cytotoxic T Cells to Murine Cytomegalovirus by Using a Nonapeptide and a Human-Compatible Adjuvant (Montanide ISA 720)

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The use of synthetic peptides representing cytotoxic T-cell (CTL) epitopes for human vaccination requires the identification of a suitable adjuvant formulation. A single immunization with Montanide ISA720/tetanus toxoid/YPHFMPTNL protected mice against murine cytomegalovirus and induced epitope-specific CTL. Such formulations will find application in peptide-based CTL anti-viral vaccines.

The development of new vaccines against a variety of diseases, particularly viral infections, in which $CD8^+$ cytotoxic T lymphocytes (CTL) play an important protective role, has been hampered by the inability of conventional vaccine formulations to induce protective CTL. A number of strategies, including immunostimulatory complexes (ISCOMS), liposomes (2), DNA (29), and virus vectors (14), have now been developed. An alternative strategy has been the use of synthetic $CD8^+$ CTL peptide epitopes as immunogens. CTL epitopes formulated with incomplete Freund's adjuvant (IFA) have been shown to induce CTL (1, 10, 12, 15, 27, 32–34) and protection (12, 15, 27, 33) in several systems. Such studies (1, 10, 15, 27, 33) often used peptides longer than the significantly more active naturally processed 8 to 10-amino-acid epitopes (23). The need for CD4 helper epitopes for CTL induction also remains controversial (17, 18, 21, 25, 30, 32, 33). There are serious reservations about widespread use of IFA in humans because of its side effects, which include granuloma formation and injection site reactions (7). We have therefore tested a series of adjuvants and formulations, currently approved or close to approval for use in humans (7), for the ability to induce CTL specific for the minimal epitope YPH FMPTNL and elicit protection against murine cytomegalovirus (MCMV). $CD8^+$ CTL directed against YPHFMPTNL, derived from an immediate-early antigen 1 pp89, represent the predominant protective response against MCMV in BALB/c mice (23, 24).

Testing of adjuvant formulations for protection against challenge with MCMV. To test adjuvant formulations (Fig. 1), mice were vaccinated (100 μl subcutaneously in the back of the neck) with a series of formulations containing YPHFMPTNL (10 μg/ml [1 μg per mouse]; Auspep Ltd., Melbourne, Australia) and were left for 2 weeks prior to challenge with MCMV. Four days after challenge spleens were removed and viral titers were determined by plaque assay (26). Tetanus toxoid (TT) was chosen to address the help requirement because of its widely accepted use and immunogenicity in humans. To mimic the situation in TT-vaccinated humans, mice were preimmunized (TT-...) 4 weeks prior to vaccination (0.03 Lyme factor per mouse in $AlPO_3$ subcutaneously [6a]). TT (0.25 μg or 0.13 Lyme factor per mouse) was then included in the vaccine (.../TT).

Protection was assessed as a significant reduction in viral titers compared with saline control animals. Mice which had recovered from a previous MCMV infection showed solid protection (MCMV, $P = 0.001$). The significant ($P = 0.02$) reduc-

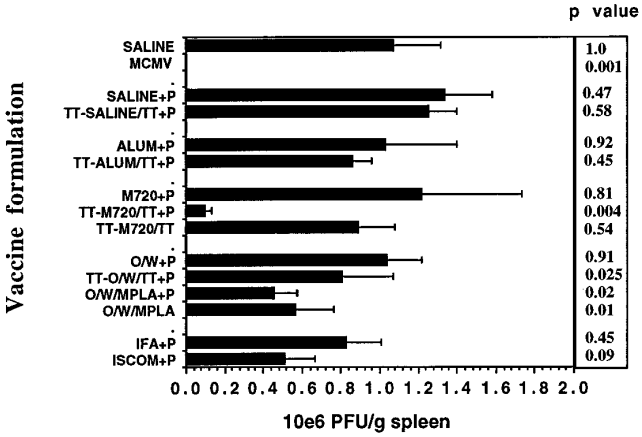

FIG. 1. Comparison of adjuvant formulations for YPHFMPTNL-mediated protection against MCMV. Viral titers ± standard errors per gram of spleen are shown from vaccinated 6 to 8-week-old BALB/c mice 4 days after MCMV challenge (K181 strain, 8×10^3 PFU, 100 μl intraperitoneally) (6, 26). Protection was assessed as a significant reduction ($P < 0.05$, unpaired Student t test) in viral spleen titers in the experimental group ($n = 5$) from those seen in the saline control ($n = 5$). Abbreviations: +P, with peptide; TT-..., preimmunization with tetanus toxoid; .../TT, TT in vaccine; MCMV, MCMV (10^4 PFU intraperitoneally)-infected cells left for 4 weeks; ALUM, peptide with or without TT absorbed onto aluminum phosphate gel (10 μg per mouse; CSL, Melbourne, Australia) overnight; M720, M720 (SEPPIC, Paris, France) vortexed with peptide in saline (7:3, [vol/vol]) with or without TT for 20 min under nitrogen gas; O/W, oil-in-water emulsion (5% squalane–1% Tween 80 in saline with or without TT, peptide, and/or MPLA (40 μg per mouse in saline; Sigma, Sydney, Australia), prepared in a Microflurodiser (Microfluidics Corp., Mass.); IFA, IFA (Sigma) formulated with saline containing peptide (7:3 [vol/vol]), emulsified by sonication; ISCOMS, ISCOMS manufactured in the presence of peptide as described previously (19). ISCOMS were then also added to peptide (10 μg/ml) in saline.

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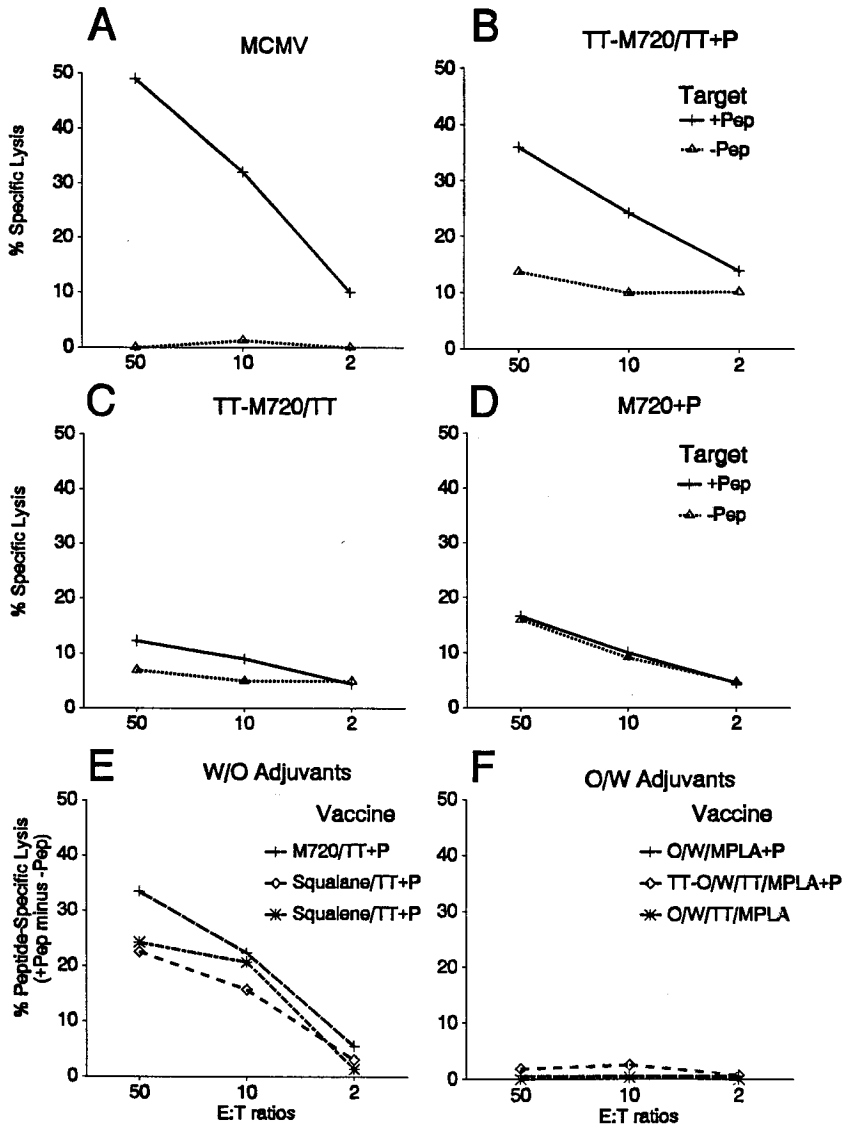

FIG. 2. Virus-specific CTL induction by the different vaccine formulations (see the legend to Fig. 1 for formulation abbreviations). Restimulated splenocytes from vaccinated animals were tested for the ability to lyse ConA T-cell blasts with or without peptide (Pep; 10 μg/ml). (A) MCMV recovered as for Fig. 1; (B) formulation showing protection in Fig. 1; (C and D) same formulation without peptide and TT, respectively; (E and F) comparison of W/O and O/W. Squalane and Squalene adjuvants, containing oil and mannide mono-oleate (4:1 [vol/vol]) (7, 11), were formulated as for M720. Percent peptide-specific lysis refers to percent lysis of peptide-sensitized ConA blasts minus percent lysis of ConA blasts without peptide, averaged for the three mice treated in parallel for each group. Standard deviations were typically <20%. For panel E, mice were not preimmunized with TT. (An M720/MPLA+P formulation failed to induce specific CTL [not shown].)

tion in spleen titers seen with O/W (oil in water)/MPLA (monophosphoryl lipid A) + P (peptide) was independent of peptide (O/W/MPLA; $P = 0.01$), illustrating that the interleukin-1 stimulator MPLA (7) induces nonspecific protection (Fig. 2). The ISCOM formulation did not protect significantly, probably because of inefficient incorporation of YPHFMPTNL into the ISCOM (8, 17, 22).

Significant peptide-mediated protection was observed only with the TT-M720 (montanide ISA 720)/TT+P formulation ($P = 0.004$) (TT-M720/TT, $P = 0.54$). In addition, no protection was achieved if TT was omitted (M720+P), illustrating an absolute requirement for help in M720 formulations containing minimal CTL epitopes (33). Preexisting TT immunity did not result in any form of carrier-induced suppression (9) or competition (4). CTL peptides can therefore easily be made immunogenic by coadministration with widely used whole-protein vaccines (like TT) originally designed to give good antibody responses. Figure 2E (M720/TT+P) also illustrated that TT pre-immunization was not required for the induction of CTL.

Demonstration of CTL in vitro. Spleen cells of BALB/c mice ($n = 3$) 4 weeks after MCMV (Fig. 2A) or 8 days after vaccine administration (Fig. 2B to D) were restimulated with MCMV-infected, UV-irradiated BALB/c 3T3 fibroblasts for 6 days (13). A standard 5-h chromium release assay was then performed with concanavalin A (ConA) blasts and ConA blasts sensitized with YPHFMPTNL as target cells. Both MCMV-recovered (Fig. 2A) and TT-M720/TT+P-vaccinated (Fig. 2B) animals produced specific CTL. Absence of either peptide (Fig. 2C) or TT (Fig. 2D) from the latter formulation resulted in no CTL activity.

Two other water-in-oil (W/O) emulsions (containing the me-

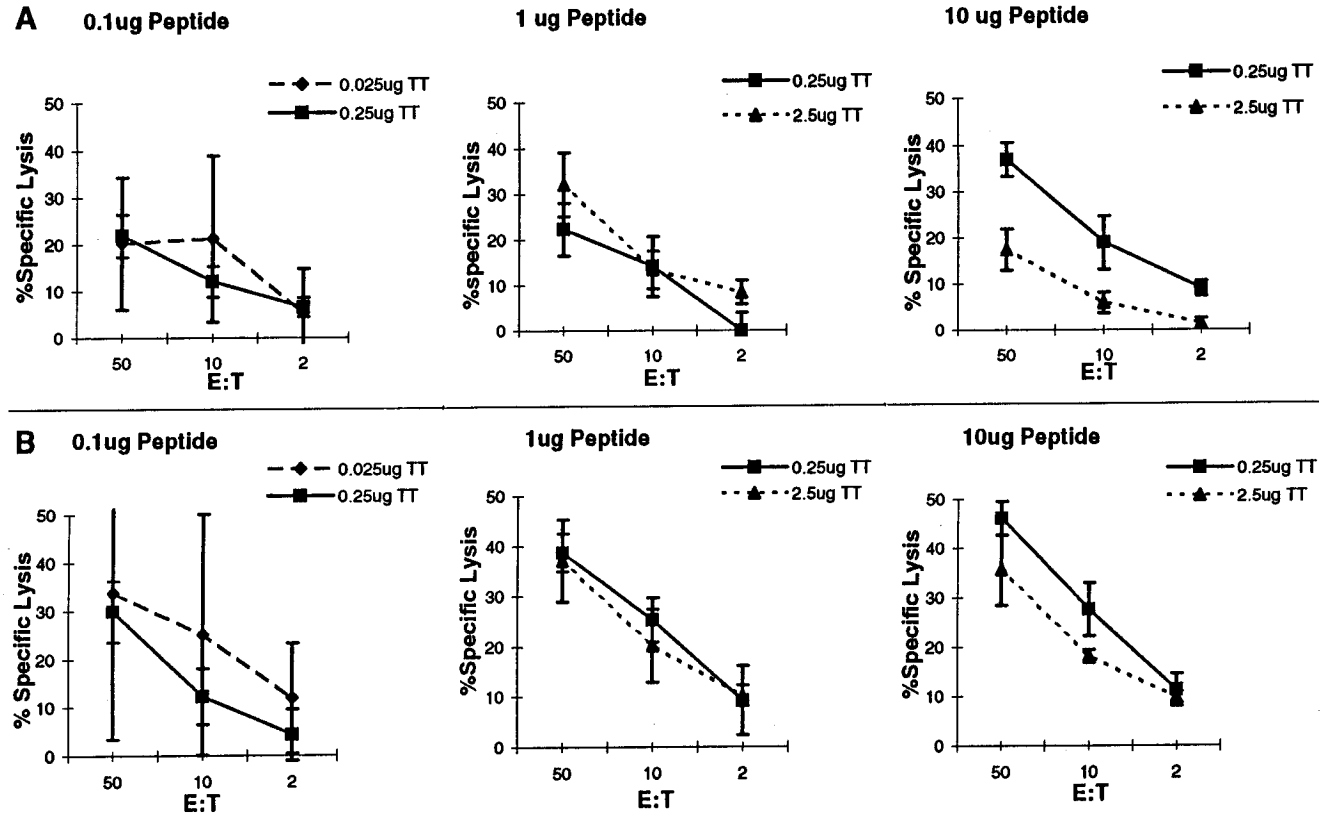

FIG. 3. Comparison of peptide and TT dose in the M720/TT+P formulation. Mice were preimmunized with TT and were then vaccinated with a series of peptide and TT concentration combinations: 0.1 μg of peptide with 0.025 and 0.25 μg of TT, 1 μg of peptide with 0.25 and 2.5 μg of TT, and 10 μg peptide with 0.25 and 2.5 μg of TT (per 100-μl inoculum). Spleen cells from each animal (n = 3 for each dose combination) were split and restimulated with MCMV/3T3 (as Fig. 2) (A) or 1 μM YPHFMPTNL (34) (B). The chromium release assay was performed as described in the legend to Fig. 2; percent peptide-specific lysis was calculated as for Fig. 2E and F. (Restimulation with peptide concentrations between 0.05 to 10 μM did not significantly alter the levels of peptide-specific lysis obtained for CTL from MCMV-infected or peptide-vaccinated mice. Peptide restimulation of spleen cells from naive mice did not induce a primary CTL response in vitro [data not shown].) E:T, effector/target cell ratio.

tabolizable oil Squalane or Squalene) gave similar CTL responses to M720 (Fig. 2E), but no O/W formulations produced specific CTL activity (Fig. 2F). W/O emulsions may be important for CTL induction because they protect the proteolytically sensitive peptide from degradation by serum proteases (31). The W/O IFA+P formulation was not effective; higher levels of peptide (34) or help (32) are required before CTLs are induced.

Effect of peptide and TT dose on the generation of CTL. A series of peptide/TT dose combinations for the TT-M720/TT+P formulation were compared (TT preimmunization remained constant) (Fig. 3A). Surprisingly, peptide dose appeared not to be critical, with broadly similar CTL responses induced over a 3-log range of peptide concentration. The 10/2.5-μg dose, however, showed a depressed response, and the 0.1/0.025-μg dose resulted in 50% of mice not responding (n = 8; data not shown). MCMV challenge studies (performed as for Fig. 1) confirmed that significant protection could be obtained for 0.1/0.25-, 1/0.025-, and 10/0.25- but not the 0.1/0.025-μg dose combination (25b).

Effect of peptide and TT dose on the generation of low-affinity CTL. Two previous reports have highlighted the potential problem of peptide vaccines inducing low-affinity CTL (5, 10), which recognize only cells sensitized with high levels of peptide, instead of high-affinity CTL, which can recognize virus infected cells naturally presenting low levels of epitope. To test this contention, parallel cultures of spleen cells from the animals in Fig. 3A were restimulated with peptide, instead of MCMV-infected 3T3 cells, and tested on the same peptide-sensitized targets as in Fig. 3A (Fig. 3B). Figure 3A (MCMV restimulated) shows high-affinity CTL, and Fig. 3B (peptide restimulated) shows high-plus-low-affinity CTL. Thus, a significantly higher level of CTL activity in Fig. 3B than in Fig. 3A would indicate that low affinity CTL were being generated at the expense of high-affinity CTL. The only dose combination for which this was readily observed was 10/2.5 μg, illustrating that induction of low-affinity CTL by peptide vaccines is not a severe complication and can also depend on the level of help.

In conclusion, this is the first description of a human-compatible W/O emulsion which can substitute for IFA for use in peptide-based CTL vaccines. A principal advantage of M720 over IFA is that it contains biodegradable oils rather than nonmetabolizable mineral oils, which are cytotoxic and implicated in the side reactions of IFA. Various components of the M720 adjuvant have been tested in humans (SEPPIC, Paris, France, confidential information; 3). Other adjuvants in the Montanide series have also been tested in human immunodeficiency virus trials (16). M720 has undergone extensive toxicology testing and a phase I trial in humans (25a). A phase I Epstein-Barr virus vaccine trial is under way with an M720/TT formulation and an Epstein-Barr virus nuclear antigen CTL epitope (20). If successful, such formulations perhaps containing multiple CTL epitopes may find wide application in the

development of human CTL peptide vaccines against viruses (2) and tumors (28).

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