Immunization with a mouse mammary tumour virus envelope protein epitope protects against tumour formation without inhibition of the virus infection

Mireille Astori and Ochine Karapetian

Swiss Institute for Experimental Cancer Research and Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland

Several immunization strategies have been proposed to inhibit tumour formation. Inactivated viral particles, purified major glycoprotein (gp52) from the viral envelope, acid-extracted subviral components, and cells or cellular extracts from tumours have all been extensively used as immunogens (Charney et al., 1976; Sarkar & Moore, 1978; Girardi et al., 1985). More recently, synthetic peptides named EP1 to EP4, derived from the primary sequence of the MMTV(C3H) major envelope protein, gp52, have been tested. These epitopes were selected because of their predicted surface accessibility. Vaccination with the EP3 peptide prior to MMTV(C3H) infection has been shown to reduce significantly the frequency of tumour formation in BALB/c mice (Dion et al., 1990).

In the studies cited above, MMTV infection accounted for the incidence of mammary gland tumours. Since then, MMTVs have been shown to encode superantigens (SAgs) (Marrack et al., 1991; Choi et al., 1991; Acha Orbea et al., 1991). MMTVs first infect B cells and then, after integration of their genome into host cells, express SAgs. These SAgs induce a transient activation and subsequent deletion of the T cells bearing particular CD4+ T cell receptor Vβ elements. The Vβ specificity depends on the polymorphic C-terminal sequence of the SAgs (Choi et al., 1992). This SAg activity parallels the early infection of the organism by MMTVs and precedes the appearance both of virus in the milk and of mammary tumours.

When MMTV(SW) is injected systemically into BALB/c mice, it triggers, as early as 5 days after injection, a strong increase in the SAg-responding Vβ6+CD4+ T cells, from 12 to 35%. On the other hand, MMTV(C3H) in similar experimental conditions induced weak proliferation, an increase of only 10 to 12% of Vβ14+CD4+-specific T cells, followed by a very slow deletion of these cells. Unlike MMTV(C3H), MMTV(SW) does not induce mammary tumours (Held et al., 1992).

We studied the effectiveness of anti-EP3 immunization on the early phases of MMTV infection by analysing SAg activity. We immunized BALB/c mice which we first infected with either MMTV(C3H), analysing late deletion of Vβ14+CD4+ T cells and the incidence of mammary tumours; or MMTV(SW), analysing early activation, the deletion of Vβ6+CD4+ T cells and the appearance of viral particles in the milk.

The mouse mammary tumour virus (MMTV) is a retrovirus transmitted from infected mothers to newborn mice during lactation (Bittner, 1942). MMTV crosses the gut via Peyer’s patches, where it infects B cells through a putative receptor before spreading to all lymphoid organs and the mammary glands (Karapetian et al., 1994). Its ‘accidental’ insertion near the host protooncogenes in the mammary gland induces carcinomas (Nusse et al., 1984). In BALB/c mice, MMTV(C3H) induces mammary tumours within 6 to 12 months, and forced breeding strongly increases tumour frequency (Hageman et al., 1981).

Author for correspondence: Mireille Astori. Present address:
Department of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland. Fax +41 21 314 07 91.
e-mail mastori@eliot.unil.ch
We first tested the protective nature of the EP3 epitope after immunization with EP3-KLH in conditions similar to those published by Dion et al. (1990). In addition to Freund’s adjuvant, we used TiterMax (CytRx, Norcross, USA). Our results with this EP3 vaccination confirmed a reduction in the incidence of tumour formation after MMTV(C3H) infection (Fig. 1a). The use of Freund’s adjuvant delayed the appearance of tumours when compared to non-immunized infected control mice, but the protection was more significant when TiterMax was used as adjuvant: it reached 80% after 1 year.

We next analysed the effect of anti-EP3 vaccination on MMTV-induced specific T cell deletion. The same immunized groups of mice used to study the incidence of tumour formation were analysed in order to measure the deletion of Vβ14+CD4+ T cells. In parallel to MMTV(C3H), a second group of immunized mice was infected with MMTV(SW). When compared to non-immunized infected control mice, those with anti-EP3-specific antibodies were not protected against the SAg-induced late deletion of Vβ14+ or Vβ6+CD4+ T cells (Fig. 1b). The anti-EP3 antibody titres measured 1 day before the infection varied between 1:450 and 1:550.

While we were able to prevent the formation of tumours, at this point we were not able to inhibit the MMTV-induced late deletion of Vβ-specific T cells. In an attempt to understand the
Epitopic immunization against MMTV

**Fig. 2.** (a) Correlation between anti-EP3 titres in the sera of mice immunized with a recombinant EP3 protein and analysis of the protection against the activation, deletion and appearance of virus in the milk after MMTV(SW) infection. Five-week-old female BALB/c mice were immunized subcutaneously with 20 µg of purified recombinant protein containing the EP3 epitope fused to four copies of the tetanus toxin T helper P30 epitope emulsified in TiterMax (Astori & Kraehenbuhl, 1996). Groups of mice with varying anti-EP3 titres were selected by ELISA the day before virus infection. The animals were infected by footpad injection with MMTV(SW). The Vβ6+CD4+ T cell percentage was determined by FACS analysis at day 5 after infection in the draining lymph node (A) and at day 60 in blood lymphocytes (B). Milk from immunized mice at their first lactation was injected into the footpad of naïve BALB/c mice and the percentage of Vβ6+CD4+ T cells in the draining lymph node was analysed 5 days later (C). Horizontal lines represent the arithmetic mean with the standard deviation of the percentage of Vβ6+CD4+ T cells in immunized but non-infected BALB/c control mice. Vertical lines show the standard deviation of the arithmetic mean of three to six mice. ND = Not detectable. (b) Analysis of the protection against MMTV(SW) infection in newborn mice passively immunized by maternal transfer of anti-EP3 immunoglobulins. Female BALB/c mice with an anti-EP3 antibody titre of 1:200,000 were bred. All F1 newborns showed anti-EP3 titres identical to their mothers on day 2 after birth. The newborns were force-nursed from day 3 to 9 and the percentage of Vβ6+CD4+ T cells in their Peyer’s patches was determined at day 9 by FACS analysis (Karapetian et al., 1994). Group 1 represents non-immunized newborn control mice fed on a non-infected BALB/c mother. Groups 2 and 3 correspond respectively to non-immunized and passively immunized newborns fed by the same MMTV(SW)-infected mother. Vertical lines show the standard deviation of the arithmetic mean of three to four mice. (c) Immunoblots and immunoprecipitation of the viral gp52 envelope protein with the serum of a mouse immunized with the recombinant EP3 protein. MMTV(C3H) or MMTV(SW) particles from milk were separated by SDS–PAGE and transferred onto nitrocellulose (panels 1 to 4). The gp52 protein was detected with a mouse anti-EP3 serum at different dilutions and the pre-immune serum (p.i.) was used as negative control (panels 1 and 2). In order to exclude a non-specific cross-reaction, serum of a mouse immunized with a recombinant protein containing an unrelated epitope [SAg ORF from MMTV(SW)] was used (panel 3). Panel 4 shows a positive control using rabbit anti-gp52 serum obtained from P. Hainaut (Université de Liège, Belgium). This figure represents results obtained from the serum of one anti-EP3 immunized mouse. Sera from other animals produced identical results. The gp52 protein from MMTV(SW)-infected milk was immunoprecipitated with a mouse anti-EP3 serum (panel 5). The pre-immune serum was used as negative control. The immune complexes were separated by SDS–PAGE and transferred onto nitrocellulose. The immunoprecipitated gp52 protein was detected with rabbit anti-gp 52 serum.

reasons for this lack of inhibition, we decided to use MMTV(SW) because of its fast SAg response and to modulate the titres of circulating antibodies. This strategy presupposed that the sequences of EP3 from MMTV(C3H) and MMTV(SW) were similar. In fact, nucleotide sequence analysis showed that the EP3 sequences of MMTV(SW) and MMTV(C3H) were identical, as presented in Fig. 1(c).

It is possible that in comparing the concentration of specific
antibody titres to the extremely potent phenomenon which produces the SAg-specific T cell response, a very small number of infecting viral particles which escaped neutralization might be enough to trigger the deletion of these T cells. To counter such a possibility, we produced a recombinant protein associating the EP3 epitope with four copies of the F30 T helper epitope from the tetanus toxin. We have shown in a previous study that this immunization strategy produces high titres of specific antibodies against the target epitope (Astori & Kraehenbuhl, 1996). Immunization of BALB/c mice with this recombinant EP3 protein produced efficient and anti-EP3-specific antibodies. In our present work, we selected groups of animals with titres ranging from 1:800 to 1:800,000. As earlier experiments had shown that the MMTV(SW)-induced activation by footpad injection was dose-dependent and followed an S-shaped curve varying from 12 to 35% (data not shown), we chose for all our protection studies a mean value of 20%, corresponding to 2

shown), we chose for all our protection studies a mean value of 20%, corresponding to 2 × 10^6 to 2 × 10^7 injected viral particles. Results from this recombinant protein vaccination showed that even very high titres of anti-EP3-specific antibodies failed to protect against induced activation and deletion of T cells by MMTV(SW) infection. Moreover, virus still appeared in the milk (Fig. 2a).

It is possible that footpad injection of MMTVs may expose a disproportionately large number of viruses to the available circulating antibodies, thereby overcoming their potential to inhibit infection. In addition, the diluting effect of the injection buffer which carries the viruses might render the antibody–virus interaction less effective. To exclude these possibilities, we fed neonates born from immunized mothers on MMTV(SW)-infected lactating mice. In these newborns, the titres of anti-EP3-specific antibodies were high and similar to those of their mothers. We have shown in a previous study that in suckling newborn mice a SAg-specific activation of Vβ6+CD4+ T cells occurs at 9 days of age in the small intestinal Peyer’s patches (Karapetian et al., 1994). The present experiment showed that infection of passively immunized newborns by the gradual delivery of MMTVs through the physiological oral route via the Peyer’s patches could not be inhibited (Fig. 2b), and that inhibition of infection could not be obtained by direct intravenous injection of viral particles in immunized adult mice with high titres of specific antibodies. This was confirmed in the latter experiment by the late deletion of SAg-specific T cells (data not shown).

To be certain that anti-EP3-specific antibodies from immunized mice were recognizing the viral gp52 envelope protein, we first performed immunoblots on MMTV particles from infected milk and then assessed by immunoprecipitation analysis the ability of anti-EP3 antibody to recognize the native gp52 protein structure on viral particles (Fig. 2c). Both experiments confirmed that the anti-EP3 antibodies from the sera of immunized mice specifically recognized the gp52 proteins on both MMTV(C3H) and MMTV(SW).

A full antibody interaction with the viral antigens might be important for inhibition of infection to occur. To test this hypothesis, we preincubated constant amounts of virus with varying titres of anti-EP3-specific antibodies before injection. Results from these experiments showed that the in vitro preincubation of viruses efficiently protected in a dose-dependent fashion animals in the early stages of the virus infection. This was proved by the absence of T cell activation. This inhibition was even more convincing when a polyclonal rabbit anti-gp52 serum was used (Fig. 3). In this case, 75% of animals were protected against the late stage of MMTV(SW) infection, as measured by the absence of deletion. This proportion reached 50% when mouse anti-EP3 serum was used (data not shown).

In the present study we have confirmed the immunoprotective role of EP3 on the incidence of mammary tumours. We were not able, however, to show any inhibition of the Vβ6-specific T cell activation or their subsequent deletion; and virus was still present in the milk.

Very high anti-EP3-specific titres obtained by recombinant protein strategy did not reverse this lack of inhibition. Preincubation experiments with the same antibodies, however, protected impressively well against the SAg responses. These results suggest that in vivo the virus has a high affinity for its receptor on B cells when compared to that of the anti-EP3 antibodies to the gp52 membrane protein. Moreover, these same preincubation experiments clearly showed that the inhibition of infection occurred before the virus–membrane interaction.

A large proportion of the MMTV envelope is made up of gp52 proteins. The preincubation certainly allows a better neutralization of these, thereby rendering inhibition more

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Fig. 3. Preincubation of MMTV(SW) with an anti-EP3 or anti-gp52 serum before infection of BALB/c mice. The percentage of Vβ6+CD4+ T cells was analysed by FACS in the popliteal lymph node of naive BALB/c mice 5 days after footpad injection of MMTV(SW) and preincubated for 30 min at 4°C with mouse anti-EP3 or rabbit anti-gp52 serum at different dilutions. Preincubation with mouse pre-immune serum (p.i.) served as negative control. Before dilution the titres of rabbit and mouse antibodies recognizing EP3 as determined by ELISA were 1:50,000 and 1:800,000 respectively. Vertical lines represent the mean value with the standard deviations of the percentage of Vβ6+CD4+ T cells in naive BALB/c mice. Horizontal lines represent the standard deviations of the arithmetic mean of three mice for each experimental condition. The experiments were repeated three times with similar results.
efficient when compared to in vivo infection. A limited number of gp52 proteins not covered by the antibodies might be sufficient to initiate the infection.

Although the development of mammary gland tumors was inhibited, anti-EP3 vaccination did not succeed in blocking virus shedding into the milk. Immunohistological observations (St George et al., 1978) have reported an uneven distribution of gp52 antigens in infected mammary glands. Hyperplastic nodules and histologically overt tumours express these antigens predominantly, whereas young hormone-induced and actively milk-secreting cells seem to exhibit them poorly. It may be that because of this difference neoplastic tissues are more susceptible to the cellular immune attack.

Transplacental transfer of maternal anti-EP3 immunoglobulins to newborns failed to protect the suckling mice infected through the physiological oral route. A reason for this could be the inaccessibility of the Peyer’s patches environment to the anti-viral antibodies (Allan & Trier, 1991).

In conclusion, we have shown, first, that the MMTV-induced tumour formation can be inhibited without the infection being neutralized. Second, we have demonstrated that the preincubation experiments were able to neutralize this virus infection and that this neutralization occurred before the virus interacted with the cell membrane.

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References


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