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Failure to Induce Protection Against Transplanted Mammary Tumours by Vaccination with the Purified Murine Mammary Tumour Virus Structural Proteins GP52 and P28*

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Abstract—*Twelve-week-old female DBAf, (BALB/c × DBAf)F1 (CD2) and GR mice were injected s.c. with the purified mouse mammary tumour virus (MTV)-derived proteins gp52 and p28 with adjuvant. Twenty days postvaccination, the DBAf mice were challenged i.p. with the MTV-containing L1210 leukaemia; the vaccinated CD2 and GR mice were challenged s.c. with solid primary mammary tumour cells. The vaccinated mice showed no delay in tumour appearance as compared to control animals which were treated with the adjuvant alone. With the leukocyte adherence inhibition assay performed on day 20 postvaccination, it was found that gp52 induced good cellular reactivity; however, factors that block cellular reactivity were also demonstrated in the serum. Antibody titers as determined by an enzyme linked immuno assay were low or negative. MTV disrupted by freezing and thawing provided slight protection in DBAf mice against the growth of the L1210 leukaemia. It is concluded that the immunogenic properties of gp52 are too weak to provide a useful vaccine against transplanted mammary tumours.*

INTRODUCTION

ALTHOUGH successful vaccination against mammary tumours has been achieved in mice with attenuated virus preparations [1–4], immunization with tumour tissue or virus can also lead to acceleration of tumour appearance [5–8]. In earlier studies we found that a protein fraction of the murine mammary tumour virus (MTV) that was enriched for the major viral membrane glycoprotein gp52 could induce some protection against either transplanted or spontaneous mammary tumours [8, 9]. However, this phenomenon proved to be highly dose-dependent, in that low doses of the vaccine evoked resistance to transplanted tumours but high doses, enhancement of tumour growth. The latter is associated with the appearance of blocking factors in the serum which interfere with cellular immune responses to MTV. There are strong indications that these blocking factors represent antigen–antibody complexes [10].

Also a mouse strain dependency was observed: strains with a high mammary tumour incidence could be somewhat protected against spontaneous tumorigenesis, while tumours appeared earlier after vaccination in mouse strains with a moderate tumour incidence and a low rate of MTV expression.

We extended these studies by immunization with highly purified MTV polypeptides: the major envelope glycoprotein with a mol. wt of 52,000 (gp52) and the major internal core protein with a mol. wt of 28,000 (p28). We investigated the induction of transplantation resistance against MTV-containing neoplasmas; to avoid influence of the adjuvant on the results [8], the control groups were treated with adjuvant alone. Cellular immune reactivity was monitored by means of the one-stage leukocyte adherence inhibition (LAI) assay; the specificity and relevance to cell

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mediated immunity in the MTV-system has been extensively established [9, 11, 12]. Anti-MTV antibodies were established by means of the enzyme-linked immunoassay (ELISA) [13].

MATERIALS AND METHODS

Animals

We used strain 2 DBAf mice which were challenged i.p. with 100 cells of the MTV-containing [14] leukaemia L1210. The effect of vaccination on mammary tumours was tested in (BALB/c × DBAf)F1 mice, further referred to as strain CD2, which were challenged s.c. with 10^4 cells derived from BALB/c mammary tumours induced by MTV-O and GR mice, which wer challenged s.c. with 10^4 cells derived from syngeneic spontaneous mammary tumours. Tumour cell suspensions were obtained as described previously [8]; before challenge, the mammary tumour cells were grown *in vitro* for 16 hr in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 10^{-5} M dexamethasone and 10 μg/ml insulin.

Antigens

Virus was purified from BALB/cfC3H mammary tumours; the viral envelope protein gp52 and the core protein p28 were isolated as described previously [15]. The purity of the proteins was near 100%. These proteins showed clear reactivity in various immuno-assays using rabbit anti-MTV serum. Before use, the purified protein preparations were subjected to dialysis. In the LAI-assay, purified virus disrupted by repeated freezing and thawing was used; murine Rauscher leukaemia virus (RLV) was purified as described [8] and treated in the same way. For the ELISA assay MTV was in addition to freeze-thawing, treated with 0.3% NNP 10.

LAI-assay

In these assays pooled cells derived from 2 animals were tested. Cellular immune reactivity was established by means of the one-stage LAI-assay as described previously [8]; RLV was used as the specificity control. Reactivity was expressed as percentage reduction in adherence of the leukocytes in the presence of MTV as compared to the RLV control value. Blocking or enhancing factors in the serum were determined by measuring the influence of serum on the adherence of immune peritoneal cells in the presence of MTV [8]; since we wanted to measure the influence of the vaccination on these serum factors, serum from untreated syngeneic age-matched mice served as a control. Blocking or enhancing capacity of serum was expressed as the quotient a: quotient $a = TS/NS$, in which TS stands for the number of adherent cells incubated with test serum and NS for the value after incubation with normal serum. Pooled test sera derived from two mice were used.

ELISA

Purified MTV was coupled by means of glutaraldehyde to 3 mm precision glass beads after the glass support had been sensitized by 3-aminopropyltriethoxysilane [13]. The antigen coated glass beads were then incubated with the test serum for 18 hr. After washing, bound antibodies were demonstrated by incubation of the glass–antigen–antibody complex with enzyme-labeled rabbit antimouse antibody. As enzyme-substrate couple, we used β-galactosidase (E.C. 3.2.1.23) and as substrate 4-methyl-umbelliferyl β-D-galactoside, which after enzymatic digestion produces the highly fluorescent product 4-methyl-umbelliferone (λ exc. = 360 nm; λ emiss. 450 nm) which is measured in the supernatant. Fluorescence was measured microfluorometrically with the same set up as formerly used for the Sepharose bead immunoassay [10]. We always tested pooled sera derived from 2 mice.

RESULTS AND DISCUSSION

Female 12-week-old DBAf mice were injected s.c. with 1 and 10 μg of either gp52 or p28. since we have indications that the structural integrity of the viral membrane in the vaccine is of importance for the resulting immune response [8], we also tested viral membrane fragments, obtained by disrupting the virus by 10 times freeze–thawing and a MTV soluble protein preparation which was obtained by dissolving virus particles in PBS containing 1% of the nonionic detergent NNP 10. As adjuvant 50 μg of an interphase material isolated from *Mycobacterium smegmatis* (IPM) was used [16]. Control animals were treated with 50 μg IPM only. In the experiment with disrupted particles NNP 10 was added to IPM in the control group. Twenty days after treatment the animals were challenged by i.p. injection of 100 L1210 cells; deaths caused by the leukaemia were recorded twice a day.

The results of the vaccination trials on

survival time of the mice are summarized in Table 1. A slight increase in survival was only observed following vaccination with 1 μg of MTV membrane fragments; no protection was observed in all other instances. Only the group treated with 10 μg gp52 showed significantly enhanced tumour growth. Sarkar and Moore [4] found that vaccination with 10 μg of purified gp52 and Freund's adjuvant protected C57BL mice against a subsequent challenge with MTV of the RIII strain. This is probably due to neutralization of the virus by male GR mice with varying doses of gp52 and p28 precipitated on alum. Control animals were treated with alum only. The results, summarized in Table 2, demonstrate that also in this experiment even when only small amounts of gp52 are administered, no clear protection against a tumour transplant was achieved. This may indicate that purified gp52 is even less immunogenic than the MTV protein fraction that was enriched for gp52 which we tested previously [8]: on that occasion, a dose of 1 μg protein precipitated on

Table 1. *Effect of injection of different MTV vaccines combined with* 50 μg *IPM in DBAf mice on the growth of* L1210 *leukaemia*

Treatment		Survival		Survival time ± S.E. in days		
Vaccine	Dose	Vaccinated	Control*	Vaccinated	Control	Significance
MTV membrane fragments	1 μg	6/10	2/10			
	10 μg	0/10	0/10	15.9 ± 0.7	15.8 ± 0.2	NS
Dissolved MTV	1 μg	1/10	4/10			
	10 μg	0/10	0/8	15.3 ± 0.4	15.1 ± 0.2	NS
gp52	1 μg	0/10	2/10			
	10 μg	0/10	0/10	14.7 ± 0.3	15.8 ± 0.2	$P<0.010$
p28	1 μg	0/9	2/10			
	10 μg	0/10	0/10	15.3 ± 0.1	15.8 ± 0.2	NS

*Controls were treated with adjuvant alone.

the induced immunologic anti-MTV reaction; they have not checked, however, the induction of transplantation resistance to tumour cells. In this latter system protection will probably be more difficult to achieve [17].

The differences in survival rates between any vaccinated and the relevant control group was not significant. However, when the animals treated with 1 μg dissolved MTV protein or with 1 μg gp52 are compared to the group that received 1 μg of MTV membrane fragments, a significant difference emerges. This indicates that virus membrane fragments induce a more effective anti-tumour immune response, and is in accord with earlier findings [4, 8].

Also in female 12-week-old CD2 mice, vaccination with different doses of purified gp52 and p28 in combination with 50 μg of IPM failed to induce protection against a mammary tumour transplant containing 10^4 cells that was given 20 days postvaccination (Table 2). In addition, we treated 12-week-old female GR mice with varying doses of gp52 and p28 precipitated on alum. Control animals were treated with alum only. The results, summarized in Table 2, demonstrate that also in this experiment even when only small amounts of gp52 are administered, no clear protection against a tumour transplant was achieved. This may indicate that purified gp52 is even less immunogenic than the MTV protein fraction that was enriched for gp52 which we tested previously [8]: on that occasion, a dose of 1 μg protein precipitated on alum caused a delay of 10 weeks in the growth of transplanted mammary tumours.

Cellular immune reactivity, the presence of factors in the serum that block or enhance cellular reactivity and anti-MTV antibody titers that were evoked by the vaccination procedures were established on day 20 postvaccination. For the DBAf mice, the results are summarized in Table 3. The control mice were negative in all tests. From these results it is clear that good cellular immune reactivity developed after all treatments. The relevance of cellular immunity induced by p28 to the *in vivo* situation remains doubtful, since this protein does not seem to be present on tumour cell membranes in large quantities, and may even be absent [18]. In the groups treated with solubilized MTV proteins and with gp52, the values of quotient *a* were significantly higher than 1.00, which indicates the presence of blocking factors. The better transplantation resistance found in the group treated with MTV membranes might be attri-

Table 2. Effect of injection of MTV proteins combined with 50 μg IPM or precipitated on alum on the growth of transplanted mammary tumours

Mouse strain	Treatment	Survival		Latency period (weeks ± S.E.)	
		Vaccinated	Controls*	Vaccinated	Controls
CD2	50 μg IPM and:				
	0.1 μg gp52	0/10	0/16	29.5 ± 2.8	24.3 ± 1.7
	1.0 μg gp52	0/10	4/10	29.8 ± 2.7	
	10.0 μg gp52	0/10			
	50 μg IPM and:				
	0.1 μg p28	0/10	0/16	24.7 ± 1.6	24.3 ± 1.7
	1.0 μg p28	0/10	4/10	29.9 ± 2.9	
	10.0 μg p28	6/10			
GR	alum and:				
	0.1 μg gp52	6/10	3/8		
	1.0 μg gp52	6/10	4/8		
	10.0 μg gp52	3.10	5/10		
	alum and:				
	0.1 μg p28	7/10	3/8		
	1.0 μg p28	4/10	4/8		
	10.0 μg p28	5/10	5/10		

*Controls were treated with adjuvant only.

*Table 3. Immunologic reactivity of DBAf mice 20 days after treatment with different MTV vaccines combined with 50 μg IPM**

Treatment		LAI percentage of reduction	Blocking-enhancing serum factors quotient *a*	ELISA positive serum dilution
MTV membrane fragments	1 μg	7‡†	0.89	—
	10 μg	20‡	1.04	—
Dissolved MTV	1 μg	6	1.20‡	—
	10 μg	12‡	1.31‡	—
gp52	1 μg	17‡	1.23‡	—
	10 μg	13‡	1.26‡	—
p28	1 μg	18‡	1.04	—
	10 μg	15‡	0.93	—

*Control animals treated with adjuvant only were negative in all tests.
‡† = Difference between test- and control-value was significant ($P < 0.010$).

buted to the absence of blocking factors that interfere with the cellular immune response in this group. These results are similar to those of our previous studies using the gp52-enriched MTV protein fraction [8].

With the ELISA no anti-MTV antibodies could be detected in the serum of the vaccinated mice, although CBA mice hyperimmunized with MTV-preparations showed a titer of 5120 in this assay. Since blocking factors were present in most sera, the low titers may be partly caused by interference of antigen–antibody complexes in the ELISA [10].

The results concerning the immunologic reactivity in vaccinated CD2 and GR mice are summarized in Table 4. In these animals, gp52 also evoked a positive cellular immune response, which was, however, counteracted by the induction of blocking factors. Only the GR mice developed anti-MTV antibodies after vaccination. From these results, it is ob-

Table 4. Immunologic reactivity of CD2 and GR mice 20 days *after treatment with different MTV vaccines**

Mouse strain	Treatment	LAI percentage of reduction	Blocking-enhancing serum factors quotient *a*	ELISA positive serum dilution
CD2	50 μg IPM and:			
	0.1 μg gp52	23‡†	1.08	—
	1.0 μg gp52	21‡	1.10	—
	10.0 μg gp52	9	1.34‡	—
	50 μg IPM and:			
	0.1 μg p28	10	1.02	—
	1.0 μg p28	13‡	0.85	—
	10.0 μg p28	14‡	1.04	—
GR	alum and:			
	0.1 μg gp52	12‡	1.10	—
	1.0 μg gp52	14‡	1.17‡	—
	10.0 μg gp52	9	1.20‡	10
	alum and:			
	0.1 μg p28	2	1.05	1250
	1.0 μg p28	11	0.94	10
	10.0 μg p28	10	1.09	250

*Control animals treated with the adjuvant alone were negative in all tests, except for the GR mice treated with alum, who had a positive anti MTV serum dilution of 10.

‡† = Difference between test- and control-value was significant ($P < 0.010$).

vious that as compared to the crude MTV protein preparation [8] purified gp52 is not a very useful vaccine for the induction of transplantation resistance. Resistance against transplanted mammary tumours is not an absolutely reliable parameter with regard to induction of resistance against primary tumour development [9]. Nevertheless, the experiments reported here indicate that the weakness of antigenicity of MTV which is common for tumour antigens becomes only more pronounced by purification.

Burton *et al.* [1] observed better transplantation resistance using irradiated tumour cells than when complete virus was employed. Stutman [7] obtained protection against spontaneous tumour development in mice treated with whole virus, but accelerated tumour development occurred after treatment with solubilized tumour cell extract. The (lipid?) environment of gp52 in the cell and MTV membrane might be an extra factor in inducing an effective immune response. Furthermore, allosteric effects also can contribute to a more immunogenic form of gp52 in case of whole tumour cells or virus as compared to purified gp52, or the cell or virus may enhance the carrier effect. However, the use of whole virus does also not exclude the possibility of tumour enhancement, which is likely to be due to the induction of antibodies which bind to antigen in the circulation and subsequently induce immune suppressive mechanisms [19]. To device vaccination procedures which circumvent the induction of blocking factors it will be mandatory to stimulate only the cellular immune response. Induction of cellular immunity depends on the structure and hydrophobicity of the antigen [20]; lipid conjugation to gp52, a procedure that is known to enhance cellular immune responses [21], might be a useful approach to achieve successful vaccination against mammary tumours.

REFERENCES

1. D. S. Burton, P. B. Blair and D. W. Weiss, Protection against mammary tumours in mice by immunization with purified mammary tumour virus preparation. *Cancer Res.* **29,** 971 (1969).
2. A. van der Gugten and P. Bentvelzen, Interference between two strains of the mouse mammary tumour virus in the GR mouse strain. *Europ. J. Cancer* **5,** 361 (1969).

3. J. Charney, J. A. Holben, C. M. Cody and D. H. Moore, Further immunization studies with mammary tumor virus. *Cancer Res.* **36,** 777 (1976).
4. N. H. Sarkar and D. H. Moore, Immunization of mice against murine mammary tumor virus infection and mammary tumor development. *Cancer Res.* **38,** 1468 (1978).
5. P. Bentvelzen, A. van der Gugten, J. Hilgers and J. H. Daams, Break through in tolerance to eggborne mammary tumour viruses in mice. In *Immunity and Tolerance in Oncogenesis.* (Edited by L. Severi) p. 525. Division Cancer Research University Perugia (1970).
6. M. M. Hirsch and I. Iversen, Accelerated development of spontaneous tumors in mice pretreated with mammary tumor tissue and adjuvant. *Cancer Res.* **21,** 752 (1961).
7. O. Stutman, Correlation of *in vitro* and *in vivo* studies of antigens relevant to the control of murine breast cancer. *Cancer Res.* **36,** 739 (1976).
8. P. Creemers, J. Ouwehand and P. Bentvelzen, Murine mammary tumor virus protein vaccine: induction of antivirus immunity and inhibition or acceleration of growth of transplanted mammary tumors. *J. nat. Cancer Inst.* **59,** 895 (1977).
9. P. Creemers, J. Ouwehand and P. Bentvelzen, Effect of a mouse mammary tumor virus-derived protein vaccine on primary tumor development in mice. *J. nat. Cancer Inst.* **60,** 1461 (1978).
10. P. Creemers and J. Brinkhof, Factors interfering with cellular immunological responses to the murine mammary tumor virus in tumor-bearing mice. *Int. J. Cancer* **20,** 441 (1977).
11. P. Creemers and P. Bentvelzen, Cellular immunity to the mammary tumour virus in mice bearing primary mammary tumours. *Europ. J. Cancer* **13,** 503 (1977).
12. P. Creemers, The role of leukocyte subpopulations in the indirect leukocyte adherence inhibition assay in the mammary tumour virus system. *Europ. J. Immunol.* **7,** 48 (1977).
13. K. Kato, H. Fukui, Y. Hamaguchi and E. Ishikawa, Enzyme-linked immuno assay: conjugation of the Fab fragment of rabbit IgG with β-D-glactosidase from *E. coli* and its use for immuno-assay. *J. Immunol.* **116,** 1554 (1976).
14. C. Radzikowski, P. Kisielow, T. Zak and A. D. Inglot, Antigenic expression of MuMTV and MuLV in DBA/2 leukaemia and DBA/2 and C3H mammary tumour cells. In *Fundamental Research on Mammary Tumours.* (Edited by J. Mouriquand) p. 137. INSERM Paris (1972).
15. F. Westenbrink, W. Koornstra and P. Bentvelzen, The major polypeptides of the murine mammary tumor virus isolated by plant-lectin affinity chromatography. *Europ. J. Biochem.* **76,** 85 (1977).
16. A. Lamensans, L. Chedid, E. Lederer, J. P. Rosselet, R. H. Gustafson, H. J. Spencer, B. Ludwig and F. M. Berger, Enhancement of immunity against murine syngeneic tumor by a fraction extracted from nonpathogenic mycobacteria. *Proc. nat. Acad. Sci. (Wash.)* **72,** 3656 (1975).
17. M. Muller and St. Zotter, Spontaneous immunity to mammary tumour virus (MTV)-associated antigens in mice and its influence on syngeneic mammary tumour growth. *Europ. J. Cancer* **8,** 495 (1972).
18. J. Yang, R. Tang and S. Nandi, Identification of the mammary tumor virus envelope glycoprotein (gp52) on mouse mammary epithelial cell surface. *Biochem. biophys. Res. Comm.* **75,** 1044 (1977).
19. P. Creemers, *Immunity to the Mouse Mammary Tumour Virus.* TNO, The Hague (1978).
20. P. C. Wilkinson, Recognition and response in mononuclear and granular phagocytes. A Review. *Clin. exp. Immunol.* **25,** 335 (1976).
21. R. L. Hunter and E. Strickland, Immunization with a lipid-conjugated membrane antigen to suppress growth of a fibrosarcoma induced by simian virus 40. *J. nat. Cancer Inst.* **54,** 1157 (1975).