

Enhancement and suppression of DTH reactivity to Rauscher murine leukaemia virus induced tumour cell lines

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Summary Delayed-type hypersensitivity (DTH) to Rauscher murine leukaemia virus (R-MuLV) encoded or induced determinants was induced in mice by three syngeneic R-MuLV-induced tumour cell lines, i.e. a myeloid tumour, RMB-1, an erythroid tumour, RED-1, and a lymphoid tumour, RLD-1. DTH to subcutaneously (s.c.) administered RMB-1 cells appeared on day 4, with a maximum DTH response on day 6 or 7. The induction of DTH could be prevented by intravenous (i.v.) pre-immunisation with R-MuLV-induced tumour cells several days before the s.c. immunisation. The three R-MuLV-induced tumour cell lines showed cross-reactivity in the DTH assay, whereas no cross-reactivity was found with syngeneic WEHI-3 cells. This indicates that the three R-MuLV-induced tumour cell lines share a virally encoded or induced antigenic determinant, which activates T-cells. When the RMB-1 cells used for immunisation had been cultured in medium supplemented with interferon- γ (IFN- γ), the subsequent DTH response was increased. This coincided with an increased expression of the R-MuLV-specific antigenic determinants on RMB-1 cells as demonstrated by Scatchard analysis. Furthermore, IFN- γ increased the MHC class I antigen expression on RMB-1 cells, whereas the class II antigen expression remained undetectable.

Immunological tumour-rejection depends on the presence of antigenic determinants on the tumour cells, which are not usually present on their normal counterparts. Such antigenic determinants may be induced by chemical, viral or physical agents and can also be found on spontaneously arising tumours (Halliday & Webb, 1969; Morton *et al.*, 1969; Kripke, 1981; Galetto *et al.*, 1985). Cells transformed by RNA tumour viruses express virally encoded proteins, and so-called virus associated proteins (Nowinski, 1978; Rogers *et al.*, 1984). These neoantigens can induce antibody formation as well as cellular immune responses, such as cellular cytotoxicity and delayed-type hypersensitivity (DTH) (Levy & Leclerc, 1977).

DTH responses can be easily elicited to various antigens, such as bacteria, viruses, xenogeneic red blood cells and contact sensitising agents (reviewed by Crowle, 1975). Tumour cells can also induce DTH (Halliday & Webb, 1969; Hawrylko, 1980; Hoover *et al.*, 1984). DTH reactions are mediated by T-cells, particularly by the L3T4 positive helper T-cell subset (Mosmann & Coffman, 1987). Evidence is increasing that Lyt-1⁺2⁻, L3T4⁺ T-cells, depending on the experimental conditions, can also mediate tumour rejection (Ozawa *et al.*, 1986; Paul *et al.*, 1987; Bookman *et al.*, 1987).

Several adjuvants have been used to increase the anti-tumour response, such as BCG (Hawrylko, 1980) and *Corynebacterium parvum* (Dye *et al.*, 1981). One could also increase the anti-tumour response by enhancing the immunogenicity of the tumour, for instance by using haptenated tumour cells (Suda *et al.*, 1986) or by treatment with interferon, which is known to increase the expression of MHC-encoded antigens (Tanaka *et al.*, 1986).

In previous studies we reported about two R-MuLV-specific monoclonal antibodies (MAbs) raised against a R-MuLV-induced myeloid tumour (Berends *et al.*, 1988a,b,c). One of the MAbs, which recognises virally encoded proteins on RMB-1 cells, was successfully used in immunotherapy. A striking difference was found between the effect of this MAb therapy in T-cell deprived nude mice and their euthymic littermates. This led us to suggest that a T-cell dependent anti-tumour immune response is involved (Berends *et al.*,

1989). Infiltration analysis of tumour foci in the liver of treated euthymic mice revealed equal numbers of L3T4⁺ and Lyt-2⁺ T-cells. However, a striking infiltrate of macrophages was present in the tumour foci of the euthymic mice. Since sensitised helper T-cells produce macrophage attracting factors, they might play an important role in the activation and influx of macrophages in the tumour foci and subsequently in tumour destruction. Since such activities are characteristic for DTH reactions, we studied whether R-MuLV-induced tumour cells indeed induced DTH in syngeneic mice.

After having established that R-MuLV-induced tumour cells indeed evoke DTH, we investigated the specificity of this response, its suppression after i.v. pre-immunisation with irradiated tumour cells, and the effect of interferon- γ (IFN- γ) on the expression of the R-MuLV-induced tumour cell surface antigens and on the immunogenicity of RMB-1 cells as measured in the DTH assay.

Materials and methods

Mice

BALB/c (H-2^d) and DBA/2 (H-2^d) female mice, 4 weeks of age, were purchased from Bomholtgard (Ry, Denmark). BALB/c (H-2^d) female mice, 4 weeks of age, were purchased from Harlan Olac Ltd (Bicester, UK). Other BALB/c (H-2^d) female and male mice were bred at the Department of Cell Biology, Immunology and Genetics of the Erasmus University.

Cell lines

Three R-MuLV-induced tumour cell lines were used: a myeloid tumour cell line of BALB/c origin (RMB-1), a lymphoid tumour cell line of DBA/2 origin (RLD-1), and an erythroid tumour cell line of DBA/2 origin (RED-1) (De Both *et al.*, 1978, 1981, 1983, 1985). The WEHI-3 immature macrophage cell line of BALB/c origin, originally described by Warner *et al.* (1969), was used as a control. This cell line does not express R-MuLV antigens. All cell lines were cultured in RPMI 1640 tissue culture medium, supplemented with 10% fetal calf serum, glutamin (4mM), penicillin

(100 IU ml⁻¹) and streptomycin (100 µg ml⁻¹), in a humidified atmosphere with 5% CO₂. IFN-γ was supplied by Drs M. van Heuvel and I.J. Bosveld as culture supernatant from a Chinese hamster cell line with the amplified murine recombinant IFN-γ gene (Dijkmans *et al.*, 1985) containing 3 × 10⁴ IU ml⁻¹. As a control culture supernatant from the CHO12RO cell line was used (Stefanini *et al.*, 1982). When IFN-γ was added to the tissue culture medium, it was supplemented to a final concentration of 100–150 IU ml⁻¹.

Antigen density on RMB-1 cells

To quantitate the density of a R-MuLV-encoded surface antigen of RMB-1 cells, a Scatchard analysis was performed, using the specific MAb 1C5F5 that was previously described (Berends *et al.*, 1988a). Briefly, RMB-1 cells were incubated for 90 min at room temperature in a volume of 100 µl of Hanks' balanced salt solution (HBSS), supplemented with 20 mM HEPES and 0.125% gelatin containing varying amounts (0.01–10 nM) of ¹²⁵I-1C5F5. Iodination of the MABs was earlier described (Berends *et al.*, 1988b). For the determination of non-specifically bound ¹²⁵I-1C5F5, the cells were incubated with varying amounts of ¹²⁵I-1C5F5 in the presence of excess (10⁻⁶ M) unlabelled 1C5F5 for 90 min. Thereafter the cells were washed to remove unbound 1C5F5. Cell-bound radioactivity was determined in an LKB 1280 ultra-gamma-counter. The association constant (*K_a*) and the number of binding sites were calculated according to Scatchard (1949).

FACScan analysis

MHC class I and class II expression of RMB-1 cells, cultured in the presence or absence of IFN-γ, was determined as earlier described (Van Ewijk *et al.*, 1981). The MABs M1/42 (Springer, 1980) and M5/114 (Bhattacharya *et al.*, 1981) that were used for the detection of class I and class II molecules, respectively, were kindly provided by the group of Dr W. van Ewijk. As a control RMB-1 cells were incubated with normal mouse serum (NMS). At least 5 × 10³ cells from each individual sample were analysed using a flow-cytofluorometer (FACScan, Becton Dickinson, Mountain View, CA, USA).

Induction of DTH reactivity

DTH was induced by s.c. immunisation of the mice with 3 × 10⁷ irradiated (20 Gy) tumour cells suspended in a volume of 300 µl. A volume of 150 µl of this suspension was injected into each inguinal area.

Assay for DTH

DTH responses were elicited by s.c. injection of 3–6 × 10⁶ irradiated (20 Gy) tumour cells, suspended in a volume of 50 µl, into the dorsum of the right hind foot 7 days after the induction of DTH. Unimmunised control mice received this challenge only. The difference in thickness of the right and left hind foot was measured 24 h later. The specific DTH response was calculated as the relative increase in foot thickness of the immunised mice minus the relative increase in foot thickness of the control mice. The increase of foot thickness of the control mice generally ranged between 10 and 20%.

Induction of suppression

Suppression of DTH was induced by i.v. administration of a high dose of heavily irradiated (80 Gy) tumour cells, 7 days before the induction of DTH. The administered number of cells is indicated in the legend to the figures.

Data analysis

For the statistical analysis of the significance of differences observed, *P* values were calculated by Student's *t* test. Values

of *P* less than 0.05 were considered significant. In Figures 1 and 3, *P* values were calculated in comparison with the increase of foot thickness of the control mice. In Figures 4 and 6 the *P* values were calculated in comparison with the specific increase of foot thickness of the relevant (positive) control group.

Results

Induction of DTH reactivity to R-MuLV-induced tumour cells

BALB/c responder mice were s.c. immunised with varying doses of syngeneic RMB-1 cells. A maximal DTH response was found after s.c. immunisation with 3 × 10⁷ RMB-1 cells (data not shown). This dose was used during all the experiments described. DBA/2 responder mice were s.c. immunised with 3 × 10⁷ syngeneic RED-1 or RLD-1 tumour cells. Seven days after s.c. immunisation all mice were challenged with 6 × 10⁶ similar tumour cells as used for immunisation. The RMB-1 cell line induced a pronounced DTH response *P* < 0.05, whereas the responses induced by RED-1 and RLD-1 cells were reproducible although not significant, *P* = 0.05 (Figure 1).

Kinetics of the DTH response to RMB-1 cells

Several groups of BALB/c responder mice were s.c. immunised with 3 × 10⁷ RMB-1 cells. At various days after immunisation, individual groups were challenged with 3 × 10⁶ RMB-1 cells. From day 4 after immunisation DTH was detectable with a maximum response around day 7 (Figure 2).

Cross-reactivity between tumour-associated antigens on different R-MuLV-induced cell lines

To investigate whether the DTH reactive T-cells responding to the R-MuLV-induced tumour cell lines recognise a common (presumably viral) determinant we investigated the cross-reactivity in the DTH assay. Thus, BALB/c mice were s.c. immunised with 3 × 10⁷ RMB-1 cells and challenged for DTH 7 days later with 6 × 10⁶ RMB-1, RED-1 or RLD-1 cells. As a control groups of BALB/c mice were challenged with 6 × 10⁶ DBA/2 spleen cells or with WEHI-3 cells. Figure 3 shows that after immunisation with RMB-1 cells challenge with each of the three tumour cell lines led to a substantial DTH response, *P* < 0.05 (lines 1–3). Challenge with DBA/2 spleen cells or with the WEHI-3 cell line, however, did not cause a DTH response suggesting the absence of cross-reactivity with non-H-2 alloantigens or other neoantigens not induced by the R-MuLV (lines 4 and 5). Cross-reactivity between the three R-MuLV-induced tumour cell lines was also found after s.c. immunisation of DBA/2 responder mice with 3 × 10⁷ RED-1 or RLD-1 cells

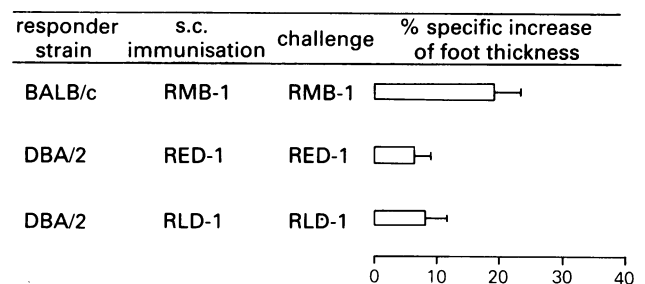


Figure 1 DTH reactivity to RMB-1, RED-1 and RLD-1 tumour cells. BALB/c mice were s.c. immunised with 3 × 10⁷ RMB-1 cells and challenged for DTH with 6 × 10⁶ RMB-1 cells 7 days later. DBA/2 responder mice were s.c. immunised with 3 × 10⁷ RED-1 or RLD-1 cells and challenged for DTH 7 days later with 6 × 10⁶ RED-1 and RLD-1 cells, respectively. Each column represents the arithmetic mean of the response ± 1 s.e.m. (*n* = 5).

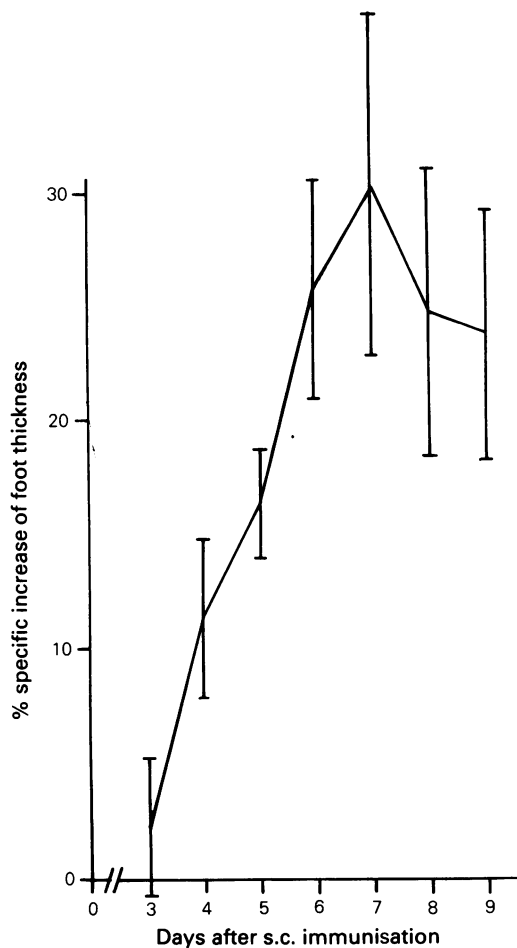


Figure 2 Kinetics of the DTH response to RMB-1 cells. BALB/c mice were s.c. immunised with 3×10^7 RMB-1 cells. DTH reactivity was determined at various intervals after s.c. immunisation. Each experimental point represents the arithmetic mean of the response ± 1 s.e.m. ($n=5$).

and challenge with RMB-1 cells, $P < 0.05$ (lines 6 and 7), whereas no DTH was found after challenge with BALB/c spleen cells (lines 8 and 9). Cross-reactivity was also determined after s.c. immunisation with RED-1 cells and challenge with RLD-1 cells and *vice versa*, $P < 0.05$ (lines 10 and 11).

Suppression of DTH to RMB-1 cells

After i.v. injection of BALB/c responder mice with either 1×10^7 or 5×10^7 irradiated RMB-1 tumour cells, s.c. immunisation with 3×10^7 RMB-1 tumour cells no longer induced a state of DTH, $P < 0.05$ (Figure 4). The suppression was found independent of whether or not the RMB-1 cells had been treated with IFN- γ . Previous studies have shown that such an i.v. pre-immunisation induces suppressor T (Ts) cells, which can suppress the subsequent induction or elicitation of DTH (Van der Kwast *et al.*, 1981; Bianchi *et al.*, 1984).

Effect of IFN- γ on antigen-expression and DTH reactivity

IFN- γ is known for its enhancing effect on the expression of various antigens. We studied the effect of IFN- γ on the expression of R-MuLV-induced antigens. To this end IFN- γ was added to the culture medium of RMB-1 cells for 24 or 48 h. Stimulation of RMB-1 cells with IFN- γ for 24 h caused a nearly two-fold increase in the amount of binding sites on RMB-1 cells detected by the binding of ^{125}I -labelled-1C5F5 (Figure 5a), and a slight decrease in affinity (Figure 5b). This coincided with a strong enhancement of DTH, $P < 0.05$

(Figure 6). After culture of RMB-1 cells with IFN- γ for 48 h the increase in R-MuLV antigen-expression was much smaller than in RMB-1 cells that had been cultured with IFN- γ for 24 h. In this case also no enhancing effect in the DTH assay was found. Apparently the IFN- γ induced increased expression of R-MuLV-encoded proteins by RMB-1 cells is only temporary. This increased antigen-expression was not due to an increase in the cell size (data not shown).

MHC class I and class II antigen expression

Since it is possible that the enhanced DTH response is (partly) due to an increased MHC antigen expression on RMB-1 cells, we also studied the MHC class I and class II antigen expression, using the monoclonal antibodies M1/42 (anti-H-2K) and M5/114 (anti-H-2I-A). From Figure 7 it is clear that class I antigens are constitutively expressed by RMB-1 cells (Figure 7a), whereas class II antigens are not (Figure 7b). Moreover, after culturing RMB-1 cells for 24 h in the presence of IFN- γ , class I antigen expression was clearly enhanced, whereas class II antigen expression remained undetectable.

Discussion

This study shows that R-MuLV-induced tumour cell lines induce DTH in syngeneic mice. This T-cell dependent response is pronounced in the case of RMB-1 cells. The weak DTH responses evoked by RED-1 and RLD-1 cells were not significant above the background level, although they were highly reproducible. This correlates with the fact that the latter two cell lines differ from the RMB-1 cell line in that they are virus-producing, whereas RMB-1 is not. Moreover, RMB-1 cells grow s.c. only after inoculation of high numbers of cells whereas RED-1 and RLD-1 cells do even after s.c. inoculation of low numbers (unpublished results).

The maximum DTH response to RMB-1 cells was found 7 days after s.c. immunisation. This is in accordance with reports by others (Hawrylko *et al.*, 1980). DTH responses to histocompatibility antigens generally peak one or two days earlier.

All three different R-MuLV-induced cell lines could induce DTH, although the responses were not equally strong. We investigated whether DTH induced by s.c. immunisation with one cell line could be elicited by challenge with another R-MuLV-induced cell line. Cross-reactivity was found in all combinations tested. The response was highest when RMB-1 cells were used for immunisation or challenge. The cross-reactivity between RED-1 and RLD-1 cells was relatively weak, as could be expected in view of the weak DTH responses these cell lines evoke. Together the results suggest that the DTH response is directed to a common R-MuLV-encoded or induced tumour-associated antigen, most clearly expressed on RMB-1 cells.

Even if tumours possess immunogenic determinants they can escape elimination by the immune system. The mechanisms involved could be the release of suppressive factors by the tumour cells (Mizel *et al.*, 1980), or the induction of suppressor cells (for a review see North, 1985). Here we report the induction of suppression of the subsequent DTH response, by administration of a high dose of heavily irradiated tumour cells. Most probably the huge amount of tumour antigens leads to the induction of the state of suppression. Previously we investigated extensively the mechanism of suppression of the DTH response to H-2 and non-H-2 histocompatibility antigens after i.v. pre-immunisation, which proved to be due to the induction of Ts cells. These Ts cells were found to be specific for the antigen(s) used for their induction (Van der Kwast *et al.*, 1981; Bianchi *et al.*, 1984). Despite this possibility of induction of antigen-specific Ts cells, it should be taken into account that factors released by the (irradiated) tumour cells

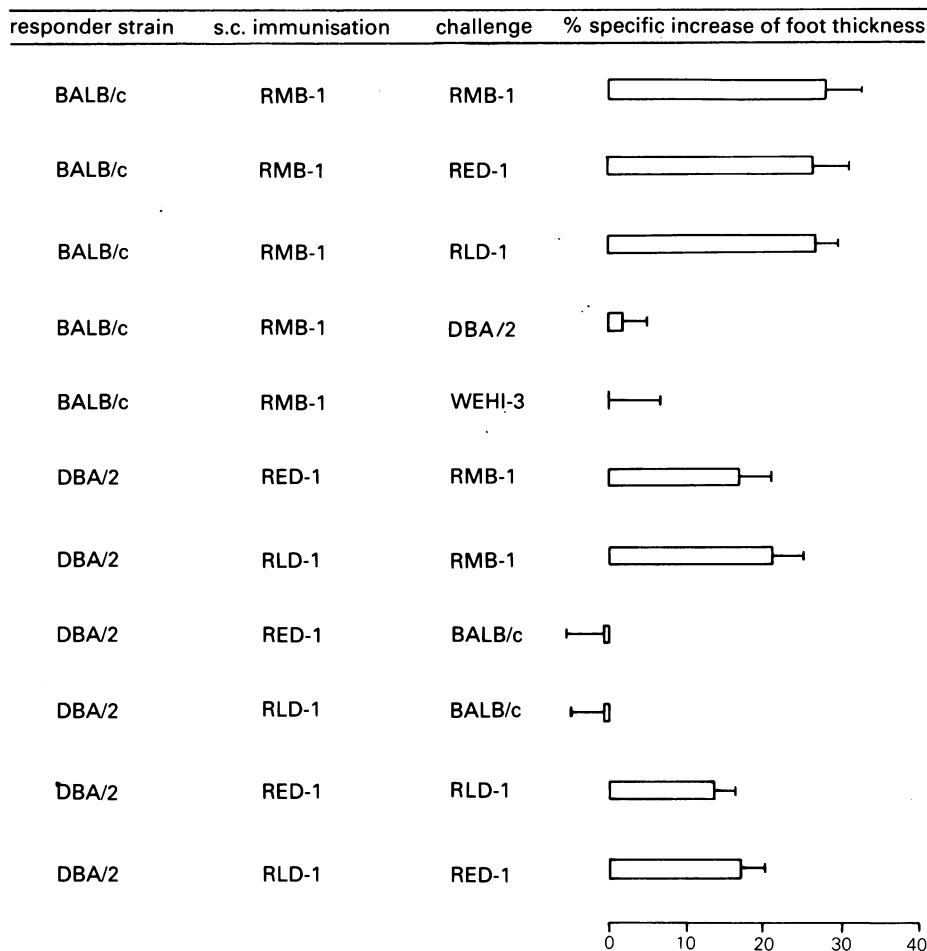


Figure 3 Cross-reactivity of tumour-associated antigens on RMB-1, RED-1 and RLD-1 cells. Several groups of BALB/c mice were s.c. immunised with 3×10^7 RMB-1 cells and challenged for DTH with 6×10^6 RMB-1, RED-1 or RLD-1 cells. Control mice were challenged with 6×10^6 DBA/2 spleen cells or WEHI-3 cells. DBA/2 responder mice were s.c. immunised with 3×10^7 RED-1 or RLD-1 cells and challenged for DTH with 6×10^6 RMB-1 cells. A DBA/2 control group was challenged with 6×10^6 BALB/c spleen cells. All cell lines had been cultured with IFN- γ for the last 24 h. Each column represents the arithmetic mean of the response ± 1 s.e.m. ($n=5$).

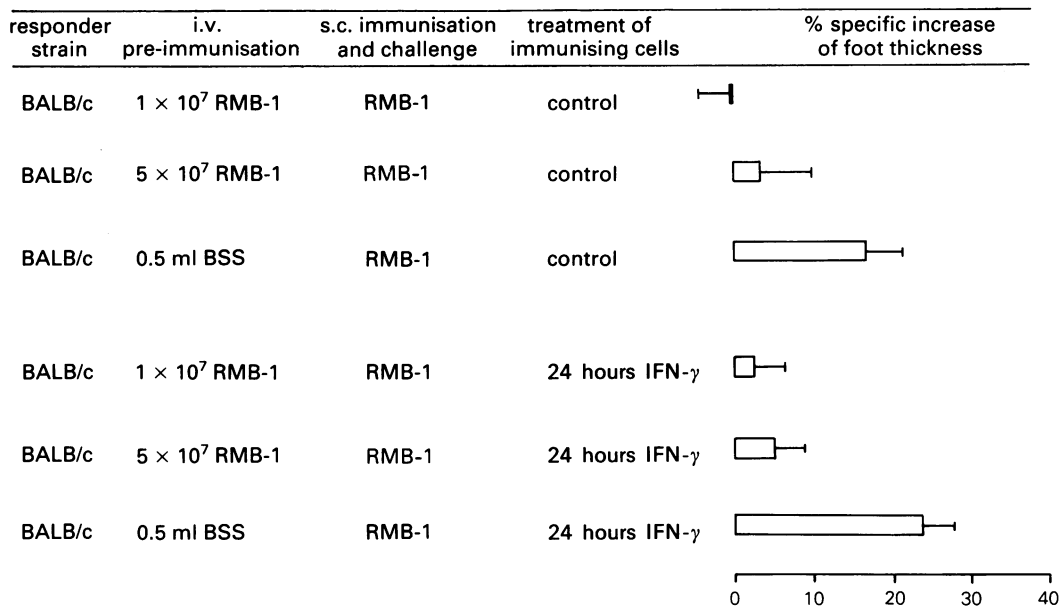


Figure 4 Induction of a state of suppression by i.v. administration of RMB-1 cells. BALB/c mice were i.v. pre-immunised with either 1 or 5×10^7 RMB-1 cells. In one experiment the RMB-1 cells used had been cultured with IFN- γ for 24 h, in the other untreated RMB-1 cells were used. Seven days after i.v. pre-immunisation all mice were s.c. immunised with 3×10^7 RMB-1 cells. Another 7 days later the mice were challenged with 6×10^6 RMB-1 cells. Each column represents the arithmetic mean of the response ± 1 s.e.m. ($n=5$).

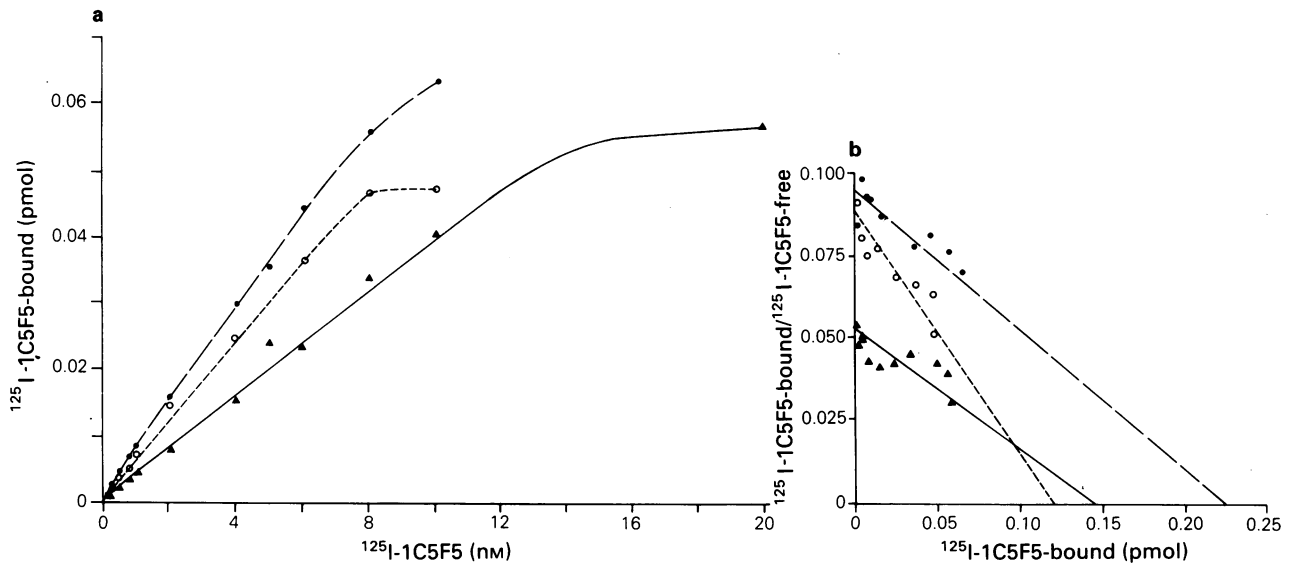


Figure 5 Determination of the number of binding sites and the association constant (K_a) of ^{125}I -labelled 1C5F5 MAbs on RMB-1 cells by Scatchard analysis. **a**, A constant number (4×10^5) of RMB-1 cells was incubated with varying amounts (0.01–10 nM) of ^{125}I -1C5F5. For the determination of non-specifically bound ^{125}I -1C5F5, the cells were incubated with excess (10^{-6} M) unlabelled 1C5F5 for 90 min, B_{max} is the number of binding sites under saturated conditions. **b**, K_a was calculated by the quotient of ^{125}I -1C5F5-bound/ ^{125}I -1C5F5-free as compared to the amount of ^{125}I -1C5F5-bound. \circ , untreated RMB-1 cells; B_{max} 180,000 cell^{-1} ; K_a 7.4×10^8 l mol^{-1} . \bullet , RMB-1 cells cultured with IFN- γ for 24 h; B_{max} 335,000 cell^{-1} ; K_a 4.2×10^8 l mol^{-1} . \blacktriangle , RMB-1 cells cultured with IFN- γ for 48 h; B_{max} 218,000 cell^{-1} ; K_a 3.6×10^8 l mol^{-1} .

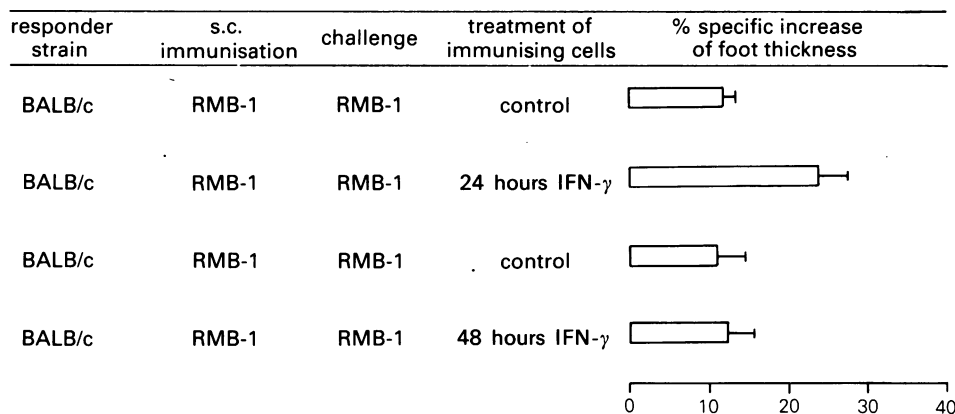


Figure 6 Effect of IFN- γ on the immunogenicity of RMB-1 cells. BALB/c mice were s.c. immunised with 3×10^7 RMB-1 cells that had been cultured with IFN- γ for 24 or 48 h. As a control mice were immunised with RMB-1 cells that had been cultured in the absence of IFN- γ . Seven days later all mice were challenged for DTH with 6×10^6 similar RMB-1 cells. Each column represents the arithmetic mean of the response \pm 1 s.e.m. ($n=5$).

might also give rise to the immunosuppression. The retroviral protein p15E should particularly be mentioned in this respect (Bendinelli *et al.*, 1985).

When suppression can be induced by a huge amount of tumour antigens presented to the immune system, this phenomenon might play a role in clinical practice, since irradiation is frequently used in the therapy of malignant tumours, which leads to massive tumour reduction, and therefore to the appearance of large amounts of tumour-derived antigens in the bloodstream. This in turn could lead

to suppression of the induction of an adequate immune response.

One approach to achieving tumour rejection is to prevent or block a possible immunosuppressive mechanism. Alternatively one might enhance the host's anti-tumour response using adjuvants, such as BCG (Hawrylko, 1980) and *Corynebacterium parvum* (Dye *et al.*, 1981). A third way would be the enhancement of tumour immunogenicity. Suda *et al.* (1986) reported increased anti-tumour immunity using haptenated tumour cells.

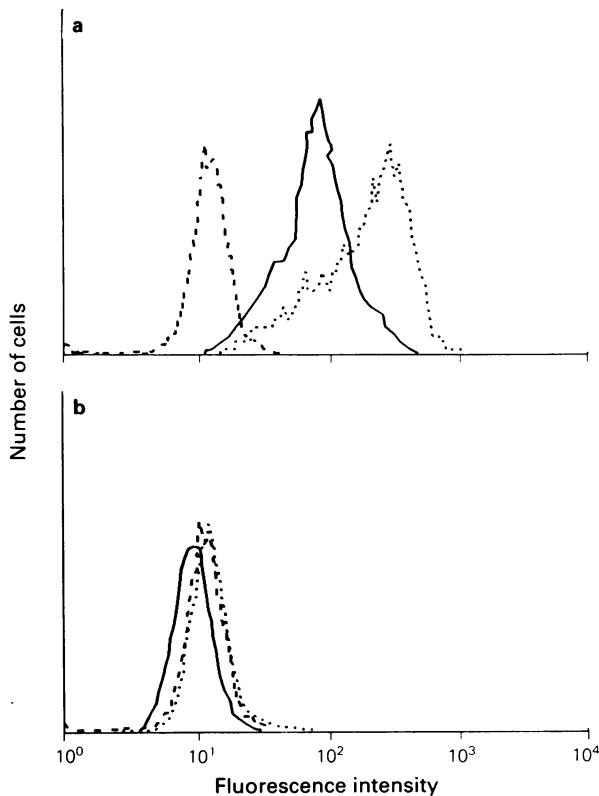


Figure 7 FACS analysis of RMB-1 cells. **a**, MHC class I expression determined using the MAb M1/42. ---- RMB-1 cells cultured with IFN- γ for 24 h, incubated with NMS as a control; — RMB-1 cells cultured in the absence of IFN- γ , stained with the MAb M1/42; RMB-1 cells cultured with IFN- γ for 24 h, stained with the MAb M1/42. **b**, MHC class II expression determined using the MAb M5/114. ---- RMB-1 cells cultured with IFN- γ for 24 h, incubated with NMS as a control; — RMB-1 cells cultured in the absence of IFN- γ , stained with the MAb M5/114; RMB-1 cells cultured with IFN- γ for 24 h, stained with the MAb M5/114.

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Here we show that IFN- γ can temporarily enhance the expression of tumour-specific antigens. Interestingly, in parallel the *in vivo* anti-tumour DTH response was enhanced (Figure 6). On one hand, the enhanced DTH response could be caused by the increased expression of R-MuLV-encoded antigens. On the other hand, it is also possible that the increased MHC class I antigen expression accounts for the increased DTH response, since the immune recognition of syngeneic tumour cells may be MHC class I restricted similarly to DTH to minor histocompatibility antigens (Van der Kwast, 1980).

Various authors asked attention for the immunomodulating effects of interferons (for reviews see Krim, 1980; De Maeyer-Guignard & De Maeyer, 1985). Interferons should exert their effects via at least three mechanisms, i.e. direct cytotoxicity, increased MHC antigen expression on the tumour cells and increased host-mediated anti-tumour effects. Most studies on the effect of interferons on tumour immunogenicity focus on the increase of the expression of MHC antigens on the tumour cells (King & Jones, 1983; Green & Philips, 1986). We found increased MHC class I antigen expression on the RMB-1 cells after culture with IFN- γ , whereas class II antigen expression remained absent. The present study shows that IFN- γ in addition enhances the expression of a virally encoded or induced tumour antigen. A similar finding has recently been reported by Greiner *et al.* (1987). They demonstrated the increased expression of a tumour-associated antigen on a human colon-xenograft after treatment with IFN- α . Accumulation of immature viral budding particles at the cell surface due to inhibitory action of IFN- α/β has been described before (Friedman *et al.*, 1980). Possibly, the effectiveness of MAb therapy of tumours can be improved by simultaneous treatment with a tumour antigen-enhancing lymphokine such as interferon.

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