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Transfer of Resistance against a Transplantable BALB/c Lymphoma*

NINA WEDDERBURN,† M. CAMPA†‡ and R. L. CARTER§

†*Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN, United Kingdom and*

§*Institute of Cancer Research and Royal Marsden Hospital, Sutton, Surrey SM2 5PX United Kingdom*

Abstract—*Resistance against an otherwise lethal challenge with a syngeneic virus-induced lymphoma (ULMC) follows prior subcutaneous (s.c.) injection of suitable doses of live or X-rayed ULMC. Partial protection followed the injection of formolized ULMC or of the inducing virus ULV given s.c. Solidly immune, but not partially protected mice, had spleen cells which could transfer resistance to recipients in a modified Winn test: serum from both groups prolonged the life of recipients of intravenous (i.v.) ULMC, and also showed complement dependent cytoxicity against ULMC* in vitro.

Lymphoma followed the i.v. but not the s.c. injection of ULV. Neither group of infected mice showed a popliteal lymph node response after footpad injection of X-rayed ULMC, or developed immune spleen cells following ULMC immunization. In addition, mice infected with ULV i.v. could not be protected from ULMC challenge by spleen cells from immune donors.

INTRODUCTION

WE PREVIOUSLY reported experiments in which the growth of a transplantable BALB/c lymphoma (ULMC) was compared in mice infected with the C type virus (ULV) which originally induced this malignant cell line, and in uninfected mice [1]. Growth of this lymphoma after i.v. injection into previously uninfected mice is almost independent of cell dose, ten cells being enough to kill half the animals, but s.c. injection of suitable doses of tumor cells can afford solid protection against subsequent i.v. challenge. Mice infected with the inducing virus ULV, given i.v., are more susceptible than untreated mice to this tumour, and s.c. injection is often followed by progressive growth.

It is unusual to find strong transplantation resistance against a lymphoma induced by a murine leukaemia virus in this strain of mice [2, 3]. We wished to determine whether protection could be produced by different immunizing schedules, in particular those which avoid the use of live malignant cells, and to correlate the resistance of the donor with the ability to transfer immunity to recipients with cells and/or serum. A modified Winn test [4] was used in which immune spleen cells or serum, and tumor cells were injected i.v. We also examined mice infected both s.c. and i.v. with ULV to determine which of the aspects of resistance to challenge with ULMC present in normal mice are altered, and to attempt to link these to the subsequent development of lymphoma.

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†Correspondence to Nina Wedderburn.

‡Present address: Institute of Microbiology, University of Pisa Italy.

MATERIALS AND METHODS

Mice

BALB/c male mice, 8–12 weeks old, were used.

Virus

Tissues from mice which had developed lymphoma following infection with ULV, a lymphomagenic C type virus originally derived from a urethane-induced leukaemia, were passed through a sieve, made up to 10% w/v in Ringer's solution, and centrifuged twice, first at 1700 *g* for 10 min, and then at

7000 *g* for 5 min, at 4°C. The final supernatant was stored at −70°C.

ULV-induced malignant cell line (ULMC)

The preparation and storage of cells from this line was described previously [1]. Cell preparations which had been passaged 70–120 times in BALB/c mice were used in these experiments. X-irradiated ULMC received 6500 rad. Formolized ULMC were treated with 4% formaldehyde in Ringer's solution at 4°C for 20 hr, and washed three times before injection.

Meth A cell line

Meth A, a 3-methylcholanthrene-induced fibrosarcoma of BALB/c mice [5] was given by Dr. L. J. Old, and passaged as an ascites form in BALB/c mice.

Anti-Thy 1.2 *serum*

This was prepared by injecting CBA thymus cells into AKR mice following the method described by Reif and Allen [6].

Spleen cell suspensions for transfer

Spleens were pressed gently through a stainless steel sieve into cold Ringer's solution. Tissue fragments were allowed to sediment. The cell suspension was spun and washed once. Viable cells were determined by nigrosine exclusion.

Modified Winn test

Spleen cells to be tested were prepared as above and injected i.v. immediately before 10^5 ULMC i.v. Each inoculum was suspended in 0.1 ml.

Popliteal lymph node (PLN) reactivity

X-rayed (6500 rad) ULMC (10^7) were injected into each hind footpad in 0.03 ml of Ringer's solution [7]. PLNs were excised at 8 days and weighed. 'Non-reactive' PLNs were not significantly heavier than controls, whereas reactive nodes weighed three or more times as much as control nodes.

Separation of T and B lymphocytes on nylon wool columns

Spleen lymphocytes were separated on Ficoll–Isopaque, suspended in Eagle's medium containing 0.01 M Hepes and 5% foetal calf serum inactivated at 56°C for 45 min, and passed through a nylon wool column. The B enriched fraction was eluted, the effluent T enriched fraction was passed through a second column before use [8].

Assay of complement dependent cytotoxicity of mouse sera

Spleens, weighing 600–800 mg from mice injected with ULMC 6–10 days previously, were taken and cell suspensions prepared as described above, in RPMI 1640 medium, (Gibco, Glasgow, U.K.), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and containing 10% foetal calf serum (Gibco, Glasgow, U.K.) which had been inactivated for 45 min at 56°C. Cell preparations with viability ≥90% were used. The assay was that described by Garrido *et al* [9]. Briefly, 7×10^4 ULMC in 100 μl of medium were added to each well of tissue-culture-grade U microtitre plates (Nunc, Denmark) with 50 μl of the serum to be tested. After 1 hr at 4°C the sera were removed amd 100 μl of rabbit serum, previously absorbed with ULMC and diluted 1 : 10, added to each well as a source of complement. After 30 min at room temperature the plates were centrifuged and the complement was removed. RPMI medium (150 μl) and 0.5 μCi of ^{3}H-thymidine (Methyl-T, Radiochemical Centre, Amersham, U.K.; spec. act., 2 Ci/mM) in 10 μl were added to each well. Plates were incubated at 37°C in a humid atmosphere of 10% Co_2, 7% O_2 and 83% N_2 for 16–18 hr. Cultures were harvested on glass fibre filter papers using an automatic harvester (Mash II, Microbiological Associates Inc.) and counted on a Packard Tri-carb liquid scintillation counter. Responses were expressed as a percentage of those observed when ULMC were treated with normal mouse serum.

RESULTS

Induction of resistance against i.v. challenge with ULMC

Preliminary experiments showed that solid protection against challenge with 10^7 ULMC i.v. could be obtained by prior inoculation of 10^5 or 10^6 of the same cells s.c. Doses of 10^2 or 10^3 ULMC given s.c. sometimes grew progressively. In order to test the possibility of including resistance without injecting live malignant cells, the effect of prior inoculation with X-rayed or formolized ULMC was in-

Table 1. Effect of various pretreatments on the susceptibility to i.v. challenge with ULMC

Immunizing* inoculum (s.c.)	Challenge dose 10^7 Deaths	10^7 MST† (±S.D.)	10^5 Deaths	10^5 MST (±S.D.)	10^3 Deaths	10^3 MST (±S.D.)
10^5 live ULMC	0/8	—	0/8	—	ND‡	
10^7 X-rayed ULMC	1/6	14.0	0/6	—	ND	
5×10^7 formolized ULMC	12/12	9.3 (±2.8)	3/12	20.3 (±1.2)	1/8	35.0
ULV	4/12	37.5 (±22.7)	3/12	13.3 (±0.6)	ND	
Nil	12/12	7.0	12/12	12.6 (±2.8)	7/8	16.4 (±2.6)

*Immunization 4 weeks before challenge.
†MST = Mean survival time (days).
‡ND = Not determined.

vestigated. We were also interested in the susceptibility or resistance of mice infected with virus by the s.c. route. Table 1 shows the results of this experiment. X-rayed ULMC gave almost complete protection. Pretreatment with formolized ULMC was ineffective against a challenge dose of 10^7 cells but protected some animals against smaller doses, while ULV given s.c. caused an increase in resistance to each challenge dose.

Transfer of resistance against ULMC challenge using spleen cells from immunized donors

The protective effect of spleen cells from animals immunized against ULMC was assayed. 'Immune' cells, and also 10^5 ULMC, were injected consecutively i.v. This dose of ULMC given i.v. is uniformly lethal when injected alone, and the survival time of recipients was not shortened by the administration of 200 mg/kg of cyclophosamide i.p. 1 day before tumour injection. In contrast, the growth of 10^5 or 10^6 s.c. injected cells was facilitated by the drug, indicating that the i.v. was less dependent than the s.c. challenge test on the immune response of the host.

When spleen cells from animals immunized with 10^5 live ULMC 4 weeks previously were tested we observed survival rates of 60–100% when cell ratios of 200–400 immune: 1 malignant cell were used in several different experiments. Normal spleen cells had no protective effect (Table 2). All survivors were immune to a second challenge with 10^7 ULMC i.v., 4 weeks later.

The ability to transfer protection by spleen cells was then compared in mice pretreated with live, X-rayed or formolized ULMC, or by ULV, given s.c. or i.v. Only cells from mice which were solidly immune (Table 1) transferred resistance (Table 2), although ratios of immune to malignant cells as high as 1000 : 1 were used.

Nature of cells transferring immunity

Spleen cells from immune donors treated with anti-Thy 1.2 serum and complement lost the ability to transfer protection. Cells were also passed twice through a nylon wool column and the T enriched fraction so obtained was tested at different immune: malignant cell ratios and was found to be 3–4 times as active as the untreated fraction on a cell: cell basis, while the B enriched fraction had no demonstrable activity.

Protective activity in the serum of immunized mice

The effect on the growth of ULMC of serum from mice pretreated in various ways was tested by injecting 0.1 ml of pooled serum together with 10^5 ULMC i.v. The results of two experiments are shown in Table 3. Almost all the recipients died, but the survival times of mice which had received serum from resistant animals were significantly lengthened. Serum from partially protected animals also had this effect in three out of four cases.

Complement dependent cytotoxicity against ULMC in sera from mice pretreated in different ways

Thymidine uptake by tumour cells was measured following incubation with antiserum and complement [9]. Treatment of ULMC with complement and anti $H2^d$ anti-serum (kindly given by Dr. W. Schmidt) or exposure of the cells to 6500 rad abolished 97% of the thymidine uptake. The batches of immune antisera used to obtain the results illustrated in Fig 1 were the same as those tested *in vivo* in experiment 2 of Table 3. Cells incubated in normal mouse serum showed the same activity as those incubated in medium alone. Sera from fully protected mice were more active in the *in vitro* test than sera from partially protected animals, and the former showed a degree of correlation between their *in vivo* and *in vitro* activity. Dilutions of from 1 : 15 to

Table 2. Protection against ULMC challenge by spleen cells from mice which had received different pretreatments

Treatment of spleen cell donors*	No. of spleen cells	Ratio spleen cells: ULMC	No. of survivors/ total	MST (days)† (range)
	None	—	0/8	10.8· (10–14)
None	10^8	1000:1	0/8	11.4 (10–13)
ULV i.v.	10^8	1000:1	0/8	10.0 (9–11)
ULV s.c.	10^8	1000:1	0/6	10.8 (10–11)
5×10^7 formolized ULMC s.c.	10^8	1000:1	0/5	11.6 (10–13)
10^7 X-rayed ULMC s.c.	10^8	1000:1	6/6	—‡
10^7 X-rayed ULMC s.c.	3×10^7	300:1	5/8	15.7 (13–19)
10^5 ULMC s.c.	6×10^7	600:1	8/8	—
10^5 ULMC s.c.	3×10^7	300:1	6/7	17.0

*Spleen cell donors pretreated 4 weeks before transfer.
†Figures in parentheses = range.
‡Survivors alive 60 days after transfer.

Table 3. Effect of serum from mice immunized in different ways on the susceptibility to i.v. challenge with 10^5 ULMC

Treatment of donors	Experiment 1 No. of survivors/ total	Experiment 1 MST (±S.D.)	Experiment 1 P*	Experiment 2 No. of survivors/ total	Experiment 2 MST (±S.D.)	Experiment 2 P
Nil	0/8	10.1 (±1.4)	—	0/8	10.8 (±1.4)	
ULV i.v.	0/8	10.5 (±1.1)	NS†	0/8	11.1 (±1.0)	NS
ULV s.c.	1/8	14.0 (±1.0)	≤0.001	0/8	12.4 (±4.0)	NS
5×10^7 formolized ULMC s.c.	0/8	13.0 (±1.7)	≤0.01	0/8	13.5 (±1.3)	≤0.002
10^7 X-rayed ULMC s.c.	ND‡	—	—	0/8	14.8 (±1.0)	≤0.001
10^5 ULMC s.c.	0/8	13.9 (±3.5)	≤0.02	1/8	14.4 (±2.5)	≤0.01

**P* value computed by Student's *t*-test, experimental group compared with group receiving serum from normal mice.
†Not significant.
‡Not determined.

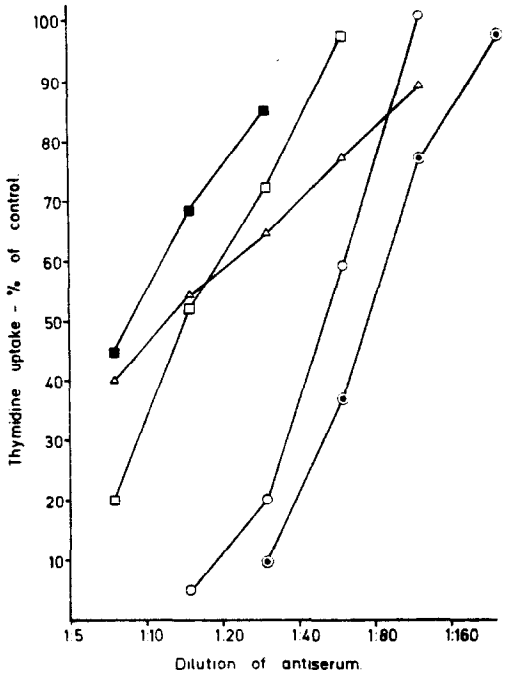

Fig. 1. Serum from donors pretreated as follows: ●—● *Live ULMC s.c.:* ○—○ *X-rayed ULMC s.c.:* ■—■ *formolized ULMC s.c.:* □—□ *ULV s.c.:* △—△ *ULV i.v.*

1 : 30 abolished 80–95% of the subsequent thymidine uptake by the cells, while the effect of injecting 0.1 ml of serum per mouse also indicated a cell killing of this order. No serum had any effect against Meth A cells *in vitro*.

Effect of the route of injection of ULV on various immune responses of infected animals, and on the development of lymphoma

We previously speculated that in mice infected with ULV i.v., two observations, namely that they are more susceptible to the growth of ULMC injected s.c. than previously untreated mice, and that they fail to show the reactivity seen in the popliteal lymph nodes (PLN) of normal mice following an injection of ULMC into the footpad, might be linked to the fact that such mice are destined to develop lymphoma [1].

We have now compared groups of animals infected with ULV by either the s.c. or the i.v. route for development of lymphoma and for PLN reactivity, and we attempted to immunize such animals against ULMC both actively and adoptively.

Groups of 12 mice were infected with ULV either s.c. or i.v. when they were 10 weeks old. All the animals which received i.v. virus developed lymphoma between 14 and 39 weeks later. None of the s.c. inoculated group became ill during this period, but one mouse became lymphomatous 46 weeks after infection; spontaneous lymphoma begins to develop in BALB/c mice during the second year of life [10].

When X-rayed ULMC were injected into the footpads of the two virus-infected groups neither showed PLN reactivity. Attempts to immunize animals given ULV i.v. with live ULMC failed because the cells grew progressively [1]: no resistance could be obtained by substituting an inoculum of X-rayed cells. The partial resistance of mice infected with ULV s.c. against ULMC challenge (Table 1) could not be increased by immunization, nor could spleen cells active in the Winn test be induced in them.

We tested the possibility of adoptively immunizing infected mice, using cells from fully immune animals. Prelymphomatous mice could not be protected against challenge even when the ratio of immune: malignant cells was 1000 : 1. Injection of immune cells protected normal mice and increased the resistance of the s.c. infected group, although this increase was not significant.

DISCUSSION

ULMC is a strongly antigenic line and is unusual in producing solid transplantation resistance in BALB/c mice. We have previously attempted to immunize this strain against several other syngeneic lymphomas, either virus-induced or spontaneous, by injection of live tumour cells, but have failed because progressive growth has occurred. Transplantation resistance can be induced easily in C57Bl mice [2] and systemic passive transfer of immunity has been demonstrated against virus-induced tumours of this strain, and also of rats [11, 12]. The lack of resistance of BALB/c mice is paralleled by a lack of cytotoxic activity in cells from mice of this strain immunized with syngeneic FV-induced tumours; congenic BALB/B and BALB/K mice, treated similarly, produce cytotoxic cells [3].

A modified Winn test [4] was used to demonstrate the transfer of resistance to ULMC. As originally described the test involves s.c. injection of tumour cells together with a population of lymphoid cells, and it can provide a better prediction of the ability of the intact animal to reject the tumour than *in vitro* cytotoxicity tests [13, 14]. However, in our system, doses of 10^4–10^6 tumour cells do not grow when injected s.c., and as smaller

doses sometimes grow progressively, interpretation of the results would be difficult, since destruction of part of the tumour inoculum might actually facilitate growth of the remainder. Other authors have partially overcome a strong immune response to the tumour by the administration of a cytotoxic drug such as cyclophosphamide one day before tumour injection [13, 15]. While the number of nucleated cells in the spleen is at a minimum at 3 days, it has recovered and exceeded the normal level 10 days after a dose of 200 mg/kg of cyclophosphamide. Since s.c. tumours may not appear until well after this time, their growth will occur in a recipient whose immune status is undergoing rapid changes during the experiment. The main disadvantage of injection by the s.c. route is that spleen cells from animals which have received a virus-containing inoculum may be antigenic in syngeneic mice [16] and may themselves provide the antigenic stimulus responsible for immunizing the recipients, in addition to transferring either immunity or suppression. Treatment of these cells in ways designed to elucidate their effector potential may also alter their antigencity.

A dose of 10^5 ULMC i.v. is uniformly lethal. The mean survival time (MST) is relatively short for such a test and is unaltered by prior administration of cyclophosphamide. However the apparent lack of effect of the untreated host immune system alone on the growth rate of this tumour dose does not preclude the possibility that it may co-operate with the transferred immune spleen cells in the induction of resistance to the challenge dose of ULMC, and also to the 100-fold larger dose against which the animals are subsequently protected. The protective effect of immune spleen cells from such mice against ULMC, when both are injected i.v. into recipients and presumably dispersed rapidly in the bloodstream in a situation in which 10 malignant cells can be lethal to an unprotected animal, is likely to depend at least partly on the action of the host's immune system against residual malignant cells. On the other hand this sort of co-operation appeared not to be effective in mice which received immune serum together with ULMC, and which showed an MST consistent with a 90% killing of ULMC, but did not ultimately survive.

Animals in which transplantation resistance can be induced easily, usually only become susceptible to virus-induced tumours after neontal rather than adult infection with virus [15, 17]. Using rats, it was shown that by varying the dose of virus, either a complete lack of response against the virus-induced tumour, or transient resistance accompanied by runting was obtained [17]. BALB/c mice given ULV i.v., on the other hand, show no signs of transplantation resistance or of autoimmunity. In fact, their skin becomes 'heterogenized', that is, it is rejected by uninfected syngeneic recipients, while they themselves do not reject skin from similar infected donors [18].

We have shown that there is also a degree of immune deficit in mice infected with ULV s.c., although they show considerable transplantation resistance to direct ULMC challenge. These mice do not have 'immune' spleen cells, and it has not proved possible to induce such cells in them.

We have also found that the PLN reactivity which follows the injection of X-rayed ULMC into the footpads of normal mice does not occur in virus infected animals, regardless of the route of injection. Reactive changes in the PLN following injection of X-rayed ULMC into normal mice are seen in both T and B cell dependent areas [14]. Similar changes occur when the differences between donor and recipient cells are such as would give rise to a mixed lymphocyte reaction, but not necessarily to the production of CTL [7].

It has been shown that the generation of cell mediated lympholytic activity against allogeneic targets involves two effector cells reacting against different antigenic determinants [19, 20]. We are at present investigating the possibility that the lymph node reactivity which we observed can be correlated with the earlier proliferative reaction which occurs during the development of cytotoxic effector cells and that the absence of such reactivity prevents the production of CTL directed against the tumour. In this case animals would either become fully immune following their first encounter with a virus-containing inoculum, or would develop an immune deficit which would prevent them from developing such immunity at a later date.

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