IN VITRO INDUCTION OF TUMOUR-SPECIFIC IMMUNITY
V. DETECTION OF COMMON ANTIGENIC DETERMINANTS
OF MURINE FIBROSARCOMAS

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Summary.—Two 3-methylcholanthrene and a spontaneous BALB/c fibrosarcoma were examined for tumour-associated antigens (TAA) by in vivo and in vitro induction of tumour-immune responses. When BALB/c mice were immunized to these fibrosarcomas by surgical tumour removal, cross-reacting tumour-associated transplantation antigens (TATA) were detected on all 3 tumours. Cytotoxic effector cells (CL) were then induced in vitro by co-culture of BALB/c spleen cells with the spontaneous, or one of the carcinogen-induced fibrosarcomas. These CL were shown to be cytotoxic T cells (Tc) and to be directed against cross-reacting TAA on all 3 tumours, by two in vitro 51Cr-release assay systems, direct 51Cr-release cytotoxicity and cellular competitive inhibition of 51Cr release. Further studies demonstrated that the fibrosarcoma TAA involved in in vivo induction of Tc were not present on normal adult or foetal tissues. A secondary cytotoxic response was also detected in vitro when spleen cells from mice immunized to a carcinogen-induced fibrosarcoma were tested. The patterns of cross-reactivity detected by the in vivo and primary in vitro tumour-immune responses suggested that the TAA detected in vivo (TATA) were not identical to the TAA detected in vitro.

The first conclusive demonstration that tumours expressed specific tumour-associated antigens (TAA) was performed by Gross in 1943, using a 3 methylcholanthrene induced C3H murine sarcoma. Subsequent experimentation in highly inbred laboratory animals has firmly established the concept of TAA and, in particular, of “unique” tumour-associated transplantation antigens (TATA) on chemically induced tumours (Foley, 1953; Prehn and Main, 1957; Klein et al., 1960; Old et al., 1962). In vivo studies with chemically induced tumours in the ensuing years have generally confirmed these observations (Baldwin, 1973; Wahl et al., 1974; Forbes, Nakao and Smith, 1975; Fritze et al., 1976). However, in a few studies, shared TATA on chemically induced tumours have also been demonstrated (Koldovsky and Svoboda, 1963; Reiner and Southam, 1967; Robert, Oth and Dumont, 1973).

The introduction of in vitro methods for the study of TAA has, however, revealed a widespread sharing of TAA among chemically induced tumours. Cross-reacting TAA have been detected in vitro both by serological techniques (Harder and McKhann, 1968; Hellstrom, Hellstrom and Pierce, 1968; Tachibana and Klein, 1970; Burdick and Wells, 1973; Fritze et al., 1976) and by assays of cell-mediated immunity where lymphoid cells from tumour-bearing or tumour-immune hosts were tested against various tumour cell lines (Hellstrom et al., 1968; Takasugi and Klein, 1970; Bataillon, Ross and Klein, 1975; Forbes et al., 1975; Whitney, Levy and Smith, 1975).

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The in vivo specificity of immunity to chemically induced tumours has been ascribed to the immunization of T lymphocytes by “unique” TATA (Rollinghoff and Warner, 1973; Kearney, Basten and Nelson, 1975; Whitney et al., 1975). The in vitro cross-reactivity of immunity, on the other hand, has been shown to vary both with the source of the effector cell (Bataillon et al., 1975) and with the timing of cell harvest after immunization (Kearney et al., 1975). The introduction of wholly in vitro methods for the induction and assay of tumour-specific immunity has provided another means of examining the TAA antigens of chemically induced tumours. McKhann and Jagarlamoody reported in 1971 the successful in vitro immunization of C3H spleen cells against 2 C3H 3-methylcholanthrene-induced fibrosarcomas. The immune lymphocytes (CL) were assayed both in vitro, on 3H-thymidine-labelled fibrosarcoma cells, and also in vivo in Winn-type assays (Klein et al., 1960; Winn, 1961) where CL admixed with tumour cells were inoculated into syngeneic mice. The in vivo Winn assay results showed clear-cut specificity of in vitro immunization to “unique” TAA on each of the tumours, but the in vitro 3H-thymidine-release assay with the same CL demonstrated some cross-reactivity between the 2 fibrosarcomas. Subsequent studies have confirmed the in vitro immunization of lymphocytes by TAA on carcinogen-induced tumours (Warnatz and Scheiffarth, 1974; Small and Trainin, 1975; Kall and Hellstrom, 1975). However, conflicting results have emerged from these in vitro experiments, with claims both that the specificity of the in vitro tumour specific immunity induced is to unique TAA alone (McKhann and Jagarlamoody, 1971; Kall, Hellstrom and Hellstrom, 1976) and that it is to both cross-reacting and unique TAA on the same tumour cells (Warnatz and Scheiffarth, 1974). The identity of the effector cytotoxic cells in these in vitro systems was not described.

This paper presents our experience with both in vitro and in vivo induction of tumour-specific immunity to 3 BALB/c fibrosarcomas, with particular reference to the TAA involved. Also included is data which indicates that the in vitro cytotoxic cell is a T lymphocyte.

MATERIALS AND METHODS

Media.—Eagle’s minimal essential medium with non-essential amino acids (Grand Island Biological Co., NY) was used supplemented with 10% foetal calf serum (FCS) (Commonwealth Serum Laboratories) 100 u/ml of penicillin, 100 μg/ml of streptomycin and buffered with sodium bicarbonate (MEMF). The medium was prepared fresh each day, and 2-mercaptoethanol (2ME) was added to a final concentration of 10^-4 M. The assay was performed in Dulbecco’s modified Eagle’s medium (Commonwealth Serum Laboratories, Melbourne, Australia) supplemented with 10% FCS (DMEM).

Animals.—Inbred male mice, aged 6–10 weeks, from the Hall Institute BALB/c An/Bradley/WEHI specific-pathogen-free colony, were used throughout this study.

Tumours.—Three BALB/c fibrosarcomas were used in this study, and were adapted to tissue culture early in their transplantation history. WEHI-164 and WEHI-167 are 3-methylcholanthrene-induced BALB/c fibrosarcomas whose origins and adaptation to tissue culture have been described previously (Rollinghoff and Warner, 1973). WEHI-11 is a BALB/c fibrosarcoma which arose spontaneously, and was adapted to tissue culture by a technique described previously (Rollinghoff and Warner, 1973). The tissue-culture lines of WEHI-164 and WEHI-11 were used for all the in vitro experiments involving these 2 tumours, whereas in the in vivo experiments the immunizations were performed with in vitro-derived cells, and the tumour challenge with either in vivo- or in vitro-derived cells. WEHI-167, however, proved difficult to maintain in tissue culture, and was therefore inoculated into BALB/c mice and maintained in serial passage in vivo. The WEHI-167 tumour cells used in this study were derived from cell suspensions prepared from in vivo tumours according to the method of von Boehmer and Shortman (1973). This technique removes dead cells and cellular debris.
from the suspension, leaving a single-cell tumour suspension of over 80% viability as determined by eosin dye exclusion. EL-4, a C57BL T lymphoma maintained in tissue culture, was used for the demonstration of specificity in the inhibition assay, and was kindly provided by Dr Alan Harris.

In vivo immunization.—The 3 fibrosarcomas were tested for the presence of TATA in BALB/c mice by the technique of surgical tumour removal as previously described (Rollinghoff, Rouse and Warner, 1973; Burton and Warner, 1976). Briefly, the minimum tumour dose (MTD) of viable tumour cells capable of initiating lethal tumour growth in 100% of inoculated BALB/c mice was determined for each tumour. Groups of BALB/c mice were then injected in the right hind leg with a dose in excess of this, and the legs amputated under ether anaesthesia when the tumour became palpable. Two weeks later these mice were challenged, together with age- and sex-matched BALB/c controls, s.c. on the right flank with the MTD of the immunizing tumour. The mice were then observed 2–3 times weekly and the tumour diameters determined by taking the mean of 2 transverse measurements of the tumour mass.

In the cross-immunity experiments, twice as many BALB/c mice were immunized by this procedure, and half challenged with the MTD of the immunizing tumour, and half with the MTD of the tumour being tested for cross immunity.

In vitro induction and assay of tumour-specific immunity.—The techniques have been previously described in detail, and so are only outlined here.

Induction procedure (Burton, Thompson and Warner, 1975).—Square 100 mm tissue-culture trays partitioned into 25 compartments (Sterlin Ltd, Richmond, Surrey, England) were used. Varying numbers of irradiated (5000 rad) stimulator fibrosarcoma cells, in a volume of 0-1 ml, were placed into the compartments and 3-6 ml of MEMF added. Then 15 × 10^6 viable nucleated responder BALB/c spleen cells (in 0-2 ml) were added to each compartment, and the trays placed in a humidified incubator at 37°C in 10% CO₂ in air for 5 days; these conditions being optimal for the induction of tumour-specific immunity. This method was adopted in order to produce responder/stimulator curves for the various in vitro direct cytotoxicity experiments. Groups of 10 identical compartments were set up for each ratio, and stimulator cell numbers, in the range of 1·5 × 10^2–1·5 × 10^6 per compartment, were used. In addition, irradiated (5000 rad) C57BL stimulator spleen cells were used to alloimmunize BALB/c responder lymphocytes in vitro by culturing the 2 cell types together as described above, but at a responder/stimulator (R/S) ratio of 10/1. BALB/c responder spleen cells alone or with 1·5 × 10^6 irradiated (5000 rad) syngeneic stimulator BALB/c spleen cells.

At the end of the incubation period the cultured cells were harvested, pelleted by light centrifugation, identical CL lots pooled, and resuspended in fresh DMEF. They were then further incubated in 35 mm Petri dishes for 21 h, as this results in a 2–3-fold augmentation of lytic activity. Finally they were harvested and a viable cytotoxic effector cell (CL) count was performed by eosin dye exclusion.

Direct ⁵¹Cr-release cytotoxic assay (Burton et al., 1975).—This was performed in quadruplicate in microtitre trays (Micro Test II Tissue Culture Plate, Falcon Plastics, Oxnard, California). Fibrosarcoma tissue-culture cells were labelled with ⁵¹Cr. Background release of ⁵¹Cr was determined by incubating 25 × 10^5 of these labelled tumour target cells in 200 μl of DMEF. The maximal amount of ⁵¹Cr releasable from the targets was assessed by lysing aliquots of 12·5 × 10^5 labelled target cells in 200 μl of Zaponin (improved lysis agent for white blood cell counts, Coulter Electronics Ltd, Dunstable, Beds., UK) in distilled water. The test assays were made at CL/target (CL/T) ratios of 100:1, 50:1 and 25:1. Thus 25 × 10^5, 12·5 × 10^5 and 6·25 × 10^5 CL in 100 μl of DMEF were dispensed into wells and then 25 × 10^3 labelled tumour target cells in 100 μl of DMEF were added.

The standard assay time was 4 h at 37°C followed by an additional hour at 45°C to facilitate the release of ⁵¹Cr from the lysed tumour target cells. Then 100 μl of supernatant was removed from each of the background and test assay wells, while from the Zaponin dilution wells 100 μl aliquots from 2 wells were pooled, since each well contained half the number of targets. These samples were counted on a Beckman Biogamma scintillation counter, and the percent specific lysis computed as
Percentage specific lysis =  
\[
\frac{\text{test count} - \text{background count}}{\text{maximal count} - \text{background count}} \times 100
\]

Inhibition assay (Chism, Burton and Warner, 1976).—The CL were first added (25 × 10^6 in 50 μl) to the test well of the microtitre tray, followed by varying numbers (in 50 μl) of unlabelled inhibitor cell preparations (blocker cells) and then the ^51Cr-labelled tumour target cells (25 × 10^3 in 100 μl). The assay conditions were then as described above for direct lysis. Control tubes assessed specific lysis in the absence of blocker cells, and the results were expressed as percent inhibition of specific lysis. In all these studies the absolute number of CL and ^51Cr target cells remained constant, only the blocker-cell number was varied, and this is given in the test figures as a blocker/target cell ratio.

Percentage inhibition of specific lysis =  
\[
\frac{\text{control lysis} - \text{test lysis}}{\text{control lysis}} \times 100
\]

Blocker cells included the 3 fibrosarcomas, EL-4, adult BALB/c viable nucleated spleen cells, and 14-day viable nucleated BALB/c foetal liver cells. The foetal-cell suspension was prepared according to a technique described previously (Chism et al., 1976).

Anti-Thy-1.2 treatment.—Anti-Thy-1.2 sera was prepared and supplied by Ms J. Gamble. Its mode of production, cytotoxic titre and specificity for T cells have been described in detail (Burton, Chism and Warner, 1976). In vitro-induced CL were treated with the serum at a dilution of 1:3 for 30 min at 37°C, after which they were resuspended in a 1:6 dilution of agarose-absorbed guinea-pig complement for a further 30 min at 37°C. The CL were then counted and made up to 25 + 10^6/ml in DMEF for the assay.

\[
\% \text{VCC} = \frac{\text{total viable CL after treatment}}{\text{total viable CL before treatment}} \times 100
\]

RESULTS

Cross immunity between fibrosarcomas in vivo

The technique of surgical tumour resection proved a reliable method for inducing tumour immunity in vivo (Table I). The use of the MTD for the challenge dose of a particular tumour allowed a clear demonstration of immunity to that tumour. Each particular tumour immunization and challenge combination was performed at least 3 times, and the results presented are from representative experiments. The first 2 lines of each section of the Table show the result of attempts to demonstrate TATA on the 3 fibrosarcomas. Both WEHI-164 and WEHI-11 regularly elicited tumour-specific immunity in the strain of origin, and in both cases there was a significant difference in the growth rate between the immunized and control mice when both groups were challenged with the MTD of the immunizing tumour (P<0·01, Student’s t test). Furthermore, ~ 50% of the mice in the groups immunized by surgical tumour removal to either of these tumours survived a challenge with the MTD of the relevant tumour, while there were no survivors in the control groups. WEHI-167, on the other hand, did not effectively immunize BALB/c mice against itself. There was no difference in the growth rate of this tumour between the mice who underwent a surgical tumour removal of WEHI-167 and the control mice, in any of the 5 experiments. In the example shown there was one survivor in the treated group, and the general finding was that survivors also were rare when mice who had undergone resection of WEHI-167 were challenged with the MTD of the tumour. Mice immunized with WEHI-164 cells do not show any significant degree of immunity to challenge with a tissue culture line of a plasmacytoma (MPC-11) indicating that cell-bound foetal calf serum antigens are probably not involved in the immunity demonstrated in Table I.

The cross-immunity experiments, however, produced some unexpected findings. In the first instance, there was strong cross immunity between WEHI-11 and WEHI-164, indicating a highly immunogenic shared TATA. Although readily detectable in reciprocal immunization and
challenge experiments, it was best demonstrated when BALB/c mice were immunized to WEHI-11 and challenged with WEHI-164. In that case the survival rate of the immunized mice was over 50% and the difference in growth rates particularly marked (P<0.01, Student's t test). The reverse situation, immunized with WEHI-164 and challenged with WEHI-11, produced fewer survivors (33%) but there was still a highly significant difference in growth rates (P<0.01, Student's t test).

When similar experiments were performed with WEHI-167, a further unexpected, but readily repeatable, result occurred. For, although mice immunized to WEHI-164 and WEHI-11 failed to reject WEHI-167, a result we had expected on the basis of the failure of WEHI-167 to immunize against itself, mice immunized to WEHI-167 regularly rejected WEHI-11. Survival rates of 50% were common when mice immunized by surgical tumour removal to WEHI-167 were challenged with WEHI-11, and there was always a significant difference in growth rates between immunized and control mice (P<0.01, Student's t test).

For WEHI-164, in contrast, there was the expected result, and mice treated by a surgical resection of WEHI-167 showed no detectable immunity to WEHI-164. This readily demonstrable cross reactivity in vivo emphasizes the need carefully to ascertain the MTD of a particular tumour in the mouse strain under investigation. Immunity due to TATA is much weaker than immunity due to histocompatibility antigens, and is easily overcome by large tumour inocula (Klein et al., 1960).

**Cross immunity between fibrosarcomas in vitro: cytotoxicity studies**

When BALB/c spleen cells are cultured under the in vitro conditions described, either alone or with varying numbers of irradiated syngeneic spleen cells, cytotoxic effector cells are induced. This phenomenon has been termed "autosensitization in vitro" (Cohen, Globerson, and Feldman, 1971; Ilfeld et al., 1973; Ilfeld, Carnaud, and Klein, 1975). We have extensively investigated this phenomenon (Burton, Chism and Warner, 1977) and so only include here illustrative data relevant to the fibrosarcomas (Table II). Here it

**Table I.** Cross Immunity in vivo between 3 BALB/c Fibrosarcomas

| Immunizing tumour | No. mice | Challenge tumour | No. survivors† | Tumour growth‡ (mean diam. (mm) ± s.e.) |
|-------------------|----------|------------------|----------------|----------------------------------------|
| WEHI-164          | 14       | WEHI-164         | 6              | 5 ± 2                                   |
| Nil§              | 9        | WEHI-164         | 0              | 19 ± 1                                 |
| WEHI-164          | 6        | WEHI-11          | 2              | 9 ± 3                                   |
| WEHI-164          | 9        | WEHI-11          | 0              | 16 ± 2                                 |
| WEHI-164          | 7        | WEHI-167         | 0              | 14 ± 1                                 |
| Nil               | 5        | WEHI-167         | 0              | 14 ± 1                                 |
| WEHI-11           | 12       | WEHI-11          | 7              | 4 ± 2                                   |
| Nil               | 9        | WEHI-11          | 0              | 12 ± 3                                 |
| WEHI-11           | 7        | WEHI-164         | 4              | 2 ± 2                                   |
| Nil               | 4        | WEHI-164         | 0              | 19 ± 1                                 |
| WEHI-11           | 6        | WEHI-167         | 1              | 16 ± 3                                 |
| Nil               | 10       | WEHI-167         | 1              | 17 ± 4                                 |
| WEHI-167          | 15       | WEHI-167         | 1              | 15 ± 6                                 |
| Nil               | 17       | WEHI-167         | 0              | 18 ± 4                                 |
| WEHI-167          | 4        | WEHI-164         | 0              | 11 ± 1                                 |
| Nil               | 7        | WEHI-164         | 1              | 11 ± 6                                 |
| WEHI-167          | 12       | WEHI-11          | 5              | 9 ± 8                                   |
| Nil               | 13       | WEHI-11          | 0              | 17 ± 4                                 |

* Mice preimmunized by surgical tumour removal.
† Mice surviving tumour-free longer than 6 weeks.
‡ Subcutaneous tumour growth as recorded at a particular time (2-3 weeks) after inoculation.
§ Age-sex-matched non-immunized mice (controls).
Table II.—Comparison of in vitro “Autosensitized” and Tumour-specific Cytotoxic Lymphocytes (Responder Spleen Cells, BALB/c)

| Stimulator* | Mean % specific lysis ± s.e. (CL/T = 100/1) | No. of expts. | 51Cr WEHI-164 | P† |
|-------------|---------------------------------------------|--------------|--------------|---|
| NIL         |                                             | 37           | 21.6 ± 2.6   |    |
| BALB/c      |                                             | 54           | 17.5 ± 1.5   | 0.24 |
| WEHI-164    |                                             | 22           | 29.5 ± 3.1   | 0.008|

* Responder/stimulator ratio 1000/1 for BALB/c CL induced in vitro to WEHI-164 and 10/1 for the “autosensitized” CL.
† P values by Mann Whitney U test between the BALB/c NIL group and the other two groups.

The cytotoxicity assays were set up at CL/T ratios of 100/1, 50/1 and 25/1. Three such experiments are shown in Fig. 1 and, as can be seen, the CL/T curve is linear in a semi-log plot. Therefore, in general, only CL/T ratios of 100/1 are reported for the cytotoxicity experiments, and in the inhibition assays the CL/T employed was 100/1, unless otherwise indicated.

The results of the in vitro experiments with the 3 fibrosarcomas revealed almost complete in vitro cross reactivity of TAA between them. In the first instance this was demonstrated by direct cytotoxicity experiments with WEHI-164 and WEHI-11. BALB/c CL induced in vitro to WEHI-164 lysed both 51Cr-labelled WEHI-164 and 51Cr-labelled WEHI-11, with the peak response to WEHI-164 at an R/S ratio of 1000/1 (Fig. 2). A similar finding occurred when BALB/c CL were induced in vitro to WEHI-11 (Fig. 3). Both 51Cr-labelled tumour targets, WEHI-11 and WEHI-164, were lysed. The peak R/S ratio, however, was 10–30-fold higher. In both cases the specific lysis of WEHI-11 was significantly less than that of WEHI-164 (P<0.01, Student’s t test, peak values). This probably reflects a difference in the rate of 51Cr release from the 2 tumours, as the inhibition experiments indicated that there was not likely to be a significant qualitative difference in TAA expression, and it has been shown that the kinetics of 51Cr release after target cell lysis by CL do vary with the cell line (Sanderson, 1976).

On the basis of these experiments, R/S ratios of 10,000/1 for WEHI-11 were chosen as optimal for the induction of CL.

Cross immunity between fibrosarcomas in vitro: inhibition studies

When BALB/c CL were induced in vitro to WEHI-164 and assayed on the same 51Cr-labelled fibrosarcoma, WEHI-
164 and WEHI-11 both inhibited lysis of the labelled target cell to the same extent (Fig. 4(a)). Normal adult BALB/c spleen cells did not inhibit lysis significantly, indicating that the antigens involved were not present on this normal tissue. In the corresponding experiment in which unlabelled WEHI-164 and WEHI-11 were added to BALB/c CL induced in vitro to WEHI-11, and ⁵¹Cr-labelled WEHI-11, there was a similar result. Both fibrosarcomas caused comparable high levels of inhibition over the blocker/target ratio range employed (Fig. 4(b)). In this second experiment, in addition to the normal spleen-cell control, viable 14-day BALB/c foetal liver cells were also added over the blocker/target ratio range shown.
However, as with the adult spleen cells, there were no significant inhibition of lysis. These results indicate that WEHI-164 and WEHI-11 share TAA as detected by these in vitro techniques, and hence confirm both the in vivo and direct in vitro cytotoxicity experiments. Furthermore, they also indicate that the TAA detected are not oncofetal or self antigens.

The final inhibition experiment was performed over a different range of blocker/target ratios, and also included WEHI-167, prepared from an in vivo tumour as described earlier. In this experiment all 3 fibrosarcomas, WEHI-164, WEHI-167 and WEHI-11, caused significant inhibition of the lysis of $^{51}$Cr labelled WEHI-164 by BALB/c CL induced in vitro to that tumour (Fig. 5). Furthermore, over this range of blocker/target ratios, differences in inhibition between the 3 tumours were apparent. For, although all 3 caused 100% inhibition at a blocker/target ratio of 30/1, indicating that there was no significant qualitative difference in TAA expression, there were significant differences in inhibition at the 3/1 blocker/target ratio. Here WEHI-164 caused 70% inhibition, but WEHI-11 only 30% ($P<0.01$ Student’s t test) which indicates a significant quantitative difference in TAA expression on these 2 tumours. It is notable that WEHI-167 is not significantly different from WEHI-164 in this respect. Again, neither 14 day BALB/c foetal liver nor normal BALB/c spleen cells caused significant inhibition, further indicating that the antigens involved are not present on these adult and foetal tissues.

The inhibition assay thus complements the direct cytotoxicity assay in the determination of the specificity of CL. However, it has a number of limitations, which we have investigated in depth (Chism et al., 1977). These include non-specific inhibition, which is particularly troublesome with large tumour cells at high blocker/target ratios. For the purposes of this paper, an inhibition assay which demonstrates that WEHI-164, the largest of the cell lines used, does not

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**Fig. 4(a).**—The inhibition curves obtained when varying numbers of unlabelled blocker WEHI-164 (○---○) and WEHI-11 (○---○) tumour cells, respectively, were added to the assay of BALB/c CL, induced in vitro with WEHI-164, and $^{51}$Cr labelled WEHI-164 (CL/T ratio of 100/1). The line ▲---▲ is the corresponding curve when adult BALB/c nucleated viable spleen cells were added at the blocker/target ratios shown.

**Fig. 4(b).**—The inhibition curves obtained when varying numbers of unlabelled blocker WEHI-164 (○---○) and WEHI-11 (○---○) tumour cells, respectively, were added to the assay of BALB/c CL, induced in vitro with WEHI-11 and $^{51}$Cr labelled WEHI-11 (CL/T ratio of 100/1).

The lines ▲---▲ and ▲---▲ are the inhibition curves obtained when viable nucleated adult BALB/c spleen and 14-day BALB/c foetal liver cells, respectively, were added to the same assay at the blocker/target ratios shown.
inhibit non-specifically at blocker/target ratios of 20/1 or less is included (Fig. 6). When BALB/c are induced in vitro to C57BL alloantigens they lyse the 51Cr-labelled C57BL T lymphoma EL-4 in vitro. When unlabelled EL-4 and WEHI-164 tumour cells are used as blockers it can be seen that EL-4 can totally inhibit lysis of the target cell, and also that WEHI-164 does not inhibit lysis over the blocker/target ratio range of 5/1 to 20/1.

**Identity of the CL induced in vitro**

The CL induced in vitro both to murine oncofoetal and plasmacytoma TAA (Rollinghoff and Wagner, 1973; Burton et al., 1976) have been shown to be T lymphocytes. When CL induced in vitro to WEHI-164 were treated with anti-

**TABLE III.—Identity of the CL induced in BALB/c in vitro to WEHI-164 (R/S = 1000/1)**

| Treatment       | % VCC* | 51Cr WEHI-164 |
|-----------------|--------|---------------|
| Nil             | 100    | 19 ± 1        |
| Complement (C1) | 82     | 22 ± 1        |
| Anti Thy-1.2    | 79     | 14 ± 1        |
| Anti Thy-1.2 + C1| 28     | 3 ± 2         |

*Viable cell count.

Thy-1.2 serum and complement (Table III) cytotoxicity was almost totally abrogated, indicating that the cytotoxicity in this system is virtually all mediated by T cells.

**Secondary cytotoxic tumour-immune response in vitro to WEHI-164**

It has previously been demonstrated that spleen cells from mice immunized in vitro to a syngeneic plasmacytoma can be induced to undergo a secondary cytotoxic tumour response in vitro (Rollinghoff, 1974) and a recent report indicates a similar secondary response to murine sarcomas (Kall et al., 1976). A group of age- and sex-matched BALB/c mice was taken and half the group immunized to WEHI-164 by surgical tumour resection. Four weeks later spleen cells were harvested from the immune and non-immune mice and cultured with irradiated WEHI-
These had expressed TAA sarcoma spontaneously, but the results may, in general, be non-immunogenic (Hewitt, Blake and Walder, 1976). However, WEHI-11, a spontaneous fibrosarcoma which arose in a BALB/c mouse, had TATA detectable in vivo and also expressed TAA in vitro. Furthermore, these tumour antigens were shared with 2 other carcinogen-induced fibrosarcomas.

As reviewed herein, cross-reacting tumour antigens have been detected on murine fibrosarcomas by both in vivo and in vitro techniques. When CL are induced in vitro to fibrosarcoma antigens it has been claimed that unique TAA dominate the response (McKhann and Jagarlamood, 1971; Warnatz and Scheif- farth, 1974; Kall and Hellstrom, 1975; Kall et al., 1976). However, in the studies presented herein, cross-reacting TAA were the major antigens detected, both in vivo and in vitro. Furthermore there is evidence that these techniques detect a heterogeneity of fibrosarcoma TAA. At least 2 cross-reacting TATA were detected in vivo, one expressed on both WEHI-11 and WEHI-164, and one expressed on WEHI-167 and WEHI-11. WEHI-167 grew equally well in mice immunized to any of the fibrosarcomas as in control mice; however, it did immunize mice to a shared TATA as detected by challenge with WEHI-11. This result illustrates a major paradox of tumour immunity, where despite the detection of TAA on many tumours a fatal outcome usually ensues for the host. A tumour may readily induce an immune response but be resistant to the effector immune mechanism. The ability of a tumour to immunize against shared TATA but grow just as rapidly in immune and non-immune mice has been reported for murine plasmacytomas (Burton and Warner, 1976). These in vivo results exemplify the need carefully to titrate tumours in the strain of origin, so that too large a challenge dose is not used.

The results of the in vitro induction of cytotoxic lymphocytes to these fibrosarcomas has demonstrated a complete sharing of antigens between the 3 tumours. The response as revealed by anti-Thy-1.2 serum treatment, is completely mediated by cytotoxic T lymphocytes, as was found for the in vivo response to the fibrosarcoma WEHI-164 (Rollinghoff and Warner, 1973). Since all 3 tumours completely inhibited the cytotoxic activity directed against one of them, a common antigenic determinant is involved. The degree of

| Table IV.—Secondary Cytotoxic Response in vitro to WEHI-164 (Stimulator Tumour Cells; WEHI-164 (5000 rad)) |
|---------------------------------------------------------------|
| **Responder spleen cells** | **Mean % specific lysis ± s.e.** |
| BALB/c | 19 ± 1‡ |
| BALB/c immune† to | 35 ± 1‡ |
| WEHI-164 | |
| * Responder/stimulator = 1000/1. |
| † Mice immunized by surgical tumour removal. |
| ‡ P<0.01, Student’s t test. |

164 in vitro at R/S = 1000/1. The CL induced were assayed on 51Cr WEHI-164, and it can be seen (Table IV) that the cytotoxicity detected in the CL induced from immune mice was about twice that of the non-immune mice. These results confirm that spleen cells from mice immune to fibrosarcomas, like those from mice immune to plasmacytomas, undergo a secondary cytotoxic response in vitro.

**DISCUSSION**

The data presented herein indicate that spontaneous and carcinogen-induced fibrosarcomas can be immunogenic both in vivo and in vitro. It has been demonstrated in vivo that the antigenicity of carcinogen-induced tumours can be correlated with the duration of the latent period and dose of carcinogen used in the induction protocol (Prehn, 1975). Tumours that appear rapidly after a high dose of carcinogen are more immunogenic than those that arise a long time after a small dose. Furthermore, it has also been shown that spontaneous malignant neoplasms of mice may, in general, be non-immunogenic (Hewitt, Blake and Walder, 1976). However, WEHI-11, a spontaneous fibrosarcoma which arose in a BALB/c mouse, had TATA detectable in vivo and also expressed TAA in vitro. Furthermore, these tumour antigens were shared with 2 other carcinogen-induced fibrosarcomas.

As reviewed herein, cross-reacting tumour antigens have been detected on murine fibrosarcomas by both in vivo and in vitro techniques. When CL are induced in vitro to fibrosarcoma antigens it has been claimed that unique TAA dominate the response (McKhann and Jagarlamood, 1971; Warnatz and Scheiffarth, 1974; Kall and Hellstrom, 1975; Kall et al., 1976). However, in the studies presented herein, cross-reacting TAA were the major antigens detected, both in vivo and in vitro. Furthermore there is evidence that these techniques detect a heterogeneity of fibrosarcoma TAA. At least 2 cross-reacting TATA were detected in vivo, one expressed on both WEHI-11 and WEHI-164, and one expressed on WEHI-167 and WEHI-11. WEHI-167 grew equally well in mice immunized to any of the fibrosarcomas as in control mice; however, it did immunize mice to a shared TATA as detected by challenge with WEHI-11. This result illustrates a major paradox of tumour immunity, where despite the detection of TAA on many tumours a fatal outcome usually ensues for the host. A tumour may readily induce an immune response but be resistant to the effector immune mechanism. The ability of a tumour to immunize against shared TATA but grow just as rapidly in immune and non-immune mice has been reported for murine plasmacytomas (Burton and Warner, 1976). These in vivo results exemplify the need carefully to titrate tumours in the strain of origin, so that too large a challenge dose is not used.

The results of the in vitro induction of cytotoxic lymphocytes to these fibrosarcomas has demonstrated a complete sharing of antigens between the 3 tumours. The response as revealed by anti-Thy-1.2 serum treatment, is completely mediated by cytotoxic T lymphocytes, as was found for the in vivo response to the fibrosarcoma WEHI-164 (Rollinghoff and Warner, 1973). Since all 3 tumours completely inhibited the cytotoxic activity directed against one of them, a common antigenic determinant is involved. The degree of
inhibition observed at blocker/target ratios of less than 30/1 is due to specific inhibition and not to the non-specific inhibition observed by these tumours at higher ratios (Fig. 6). The nature of this common antigenic determinant has not been fully defined, but is clearly shown to be distinct from the oncofoetal antigens also expressed by these tumours (Chism et al., 1976) since foetal liver shows no significant blocking in this system, whereas it does so in the OFA studies (Chism et al., 1976).

The possibility that sensitization to foetal calf serum components is involved has been discussed elsewhere in detail (Burton et al., 1977) but is not the explanation for this common antigen, since the WEHI-167 cells were derived from in vivo tumour, not from a cultured line.

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