Europ. J. Cancer Vol. 15, pp. 537–542.
Pergamon Press Ltd. 1979. Printed in Great Britain

0014–2964/79/0401–0537 $02.00/0

Tumour Sensitization Obtained by Injection of Antigenic Tumour Cells into the Caecal Lumen of Syngeneic Mice*

M. LAU LAURSEN and KIRSTEN LAURSEN
The Fibiger Laboratory, Copenhagen, Denmark

Abstract—*Syngeneic viable malignant ascites cells inoculated into the caecal lumen of C3H mice afforded protection against a subsequent intraperitoneal graft of the same tumor cells. Cell mediated as well as humoral-mediated immunity could be demonstrated by* in vitro *tests after lumenal intracaecal (i.c.) inoculation of the ascites cells. The immunity obtained by the i.c. route could be transferred to untreated animals by immune spleen cells and by immune serum. The effect of immune spleen cells was inhibited by treatment of the recipients with cyclophosphamide. In the presence of antigen, adoptive transfer of spleen-cell-mediated immunity induced production of specific antibodies. The spleen was not imperative for sensitization by the i.c. route.*

INTRODUCTION

THE GASTROINTESTINAL tract has an extensive mucosal surface with the capacity to absorb a variety of nutrient substances. In recent years it has been recognized that the gut is an importent lymphoid organ. Various types of lymphoid cells are found in the mucosa of the intestinal tract [1]. There is evidence that antigen within the lumen of the gut can stimulate an immune response [2–4].

Very little is known about the local immune response from the gut to tumor cells. However, by inoculating malignant cells into the intestinal lumen of the rat Lund [5] found protection against a subsequent tumor graft. The present authors have also reported [6, 7] that antigenic tumour cells injected into the lumen of caecum of syngeneic mice caused either protection against, or enhanced growth of, a subsequent tumour graft, depending on the state of the cells inoculated intracoecally (i.c.).

Whether the immunity obtained by i.c. immunization was by cell-mediated or by humoural mediated factors, however, remained an open question.

The purpose of the present study was to investigate the immunity leading to protection against a subsequent isograft following i.c. injection of the tumour cells.

Accepted 15 September 1978.
*This work was supported in part by The Danish Medical Research Council.
Correspondance to: M. Lau Laursen, The Fibiger Laboratory, Ndr. Frihavnsgade 70, DK-2100 Copenhagen, Denmark.

MATERIALS AND METHODS

Eight- to ten-week-old inbred mice of the C3H Fib strain were used in this work. The animals were reared and kept under minimal disease conditions with up to 10 mice in each cage. The mice were maintained on a sterilized pellet diet and filtered water *ad libitum*.

The malignant cell line was derived from cultures of C3H mouse lung fibroblasts which had undergone spontaneous malignant conversion and transformation during propagation *in vitro*. From these cells an ascites tumour (C3H-L 1/a) was established which grew equally well *in vivo* and *in vitro* [8]. The ascites cells have previously been shown to possess isoimmunizing properties [6, 8, 9].

The caecum in the mouse represents a convenient target for injection of cells; it is relatively large and quite mobile. Immunization by this route required a small laparotomy of ether-anaesthetized mice. The caecum was drawn out of the abdominal cavity and the desired number of cells, suspended in a volume of 0.1 ml PBS, were inoculated into the caecal lumen with a small injection needle (27G × 1).

Serum was prepared from blood obtained by retro-orbital bleeding. The blood was al-

Table 1. Immune response to malignant ascites cells inoculated into the caecal lumen of C3H mice

Active protection test*			Indirect immuno-fluorescence test†		
No. of C3H-L 1/a cells inoculated i.c.	Dead with tumours/group	Survival for tumour-takes ($\bar{x} \pm s$ days)	Fluorescent index Serum dilutions 1:2	1:8	1:32
10^5	12/15	19.2±3.3	0.26	0.30	0.08
10^6	13/15	22.0±3.3	0.57	0.41	0.10
10^7	1/15		0.79	0.74	0.42
10^8	0/15		0.92	0.92	0.84
0 but PBS	9/10	21.3±2.4			
0	8/10	20.3±2.0			

According to the Fourfold Table Test, groups receiving 10^7 and 10^8 cells twice by i.c. differ significantly at the 99 per cent level from the other groups.

*Each mouse received two immunizing injections of C3H-L 1/a cells, separated by an interval of one week, into the coecal lumen. Two weeks later they were challenged with 5×10^6 C3H-L 1/a cells i.p.

†Antisera were prepared from blood samples withdrawn immediately before the i.p. challenge.

lowed to coagulate at room temperature. The clot was carefully detached from the glass and removed by centrifugation. The serum was stored at minus 70°C. After bleeding the mice were killed by cervical dislocation. Spleens and lymph nodes were removed and single cell suspensions were prepared by scissoring and further disruption in a loose-fitting glass homogenizer. Lymphocytes from the intestinal wall (caecum and the proximal part of the colon) were prepared in the same way but, before cutting, the tissue was washed carefully in PBS. In the trypan blue dye exclusion test more than 80% of the cells proved to be viable.

Specific antibodies directed against the tumour cell membrane were demonstrated by the indirect immuno-fluorescence technique [10], using live cells as target cells. Staining was performed with polyvalent fluorescein-conjugated antimouse gammaglobulin, purchased from Nordic, Holland.

The fluorescent index was calculated as $(a-b)/a$, where a = the percentage of fluorescent negative cells treated with normal control serum, and b = the percentage of fluorescent negative cells treated with the serum to be tested [11].

The microcytotoxicity test [12] was carried out in plastic plates No. 1480 from NUNC, Denmark. Before use the plates were bathed in absolute alcohol and sterilized by u.v. rays for 30 min. Target tumour cells were pipetted into the wells (100 cells/well) in tissue culture medium Fib 41B [8] containing 20% fetal calf serum. After 20 hr of incubation at 37°C the medium was decanted. Lymphocyte suspensions were washed 4 times and added at lymphocyte:target cell ratios of 50:1. The plates were incubated for 42 hr at 37°C, after which the medium with unattached cells was decanted and the plates washed twice with saline. Cells were stained with Giemsa and the number of cells per well counted under a microscope.

RESULTS

Table 1 shows the effect of different numbers of C3H-L/a tumour cells inoculated twice, separated by an interval of one week, into the caecal lumen. Injection of PBS containing 10^5 or 10^6 cells did not induce protection against an intraperitoneal (i.p.) graft of the same cells 14 days later, whereas injection of 10^7 and 10^8 cells intracoecally (i.c.) provided a significant protection against the later i.p. graft.

From Table 1 it is further seen that a dose-dependent antibody response to i.c. injected C3H-L/a cells was revealed by the indirect membrane fluorescence test, using pooled sera.

Table 2 shows the results of the microcytotoxicity *in vitro* test for cell-mediated immunity. The lymphocyte donors were immunized 12 days before the test by the i.c. route. The target cells were at the same time explanted from tumour-bearing animals and the test was carried out at the fifth passage of the cells *in vitro*.

The table shows that lymphocytes from the intestinal wall and the mesenteric lymph node

Table 2. Cytotoxic effects of C3H lymphoid cells (LNC) on C3H-L 1/a cells in vitro§

Effector cells	Mean number of target cells ±SD left in 16 wells *a* Non-immune	*b* Immune¶	Cytotoxic index‖ (%)
Axillary and inguinal LNC	72±11.3	55±7.4†	24
Spleen cells	77±8.9	61±11.4*	21
Mesenteric LNC	72±9.2	44±9.0‡	39
Lymphocytes from intestinal wall	70±6.7	35±12.6‡	50
Effector cells from mice immunized subcutaneously			
Axillary LNC	72±11.3	35±6.1‡	51
Non-treated targetcells without LNC	83±9.7**		

*$P<0.05$ †$P<0.005$ ‡$P<0.001$ (Students *t*-test).
§Lymphocyte:target cell ratio = 50:1.
‖Cytotoxic index = $(a-b)/a \times 100$.
¶Immunized by a single intracaecal injection of 10^7 C3H-L 1/a cells. Lymphoid cells harvested 12 days later.
**Significantly higher than controls treated with non-immune ($P<0.05$) and immune lymphocytes ($P<0.001$).

cells had a significantly higher degree of cytotoxicity than spleen cells and axillary lymph node cells ($P<0.05$). After exposure to immune lymphocytes from the gut the mean number of the target cells was reduced by 50%, when compared with target cells treated with non-immune lymphocytes. Immune spleen cells reduced the number of target cells by 21%.

It has previously [6] been shown that the protective effect obtained by i.c. immunization with live ascites cells could be transferred to untreated animals by immune spleen cells. Table 3 confirms this finding, but the table further shows that the protective effect was prevented by treatment of the recipients with 5 mg cyclophosphamide.

Table 3. Influence of cyclophosphamide (CPA) on the protective effect of immune spleen cells against a simultaneous i.p. graft with 10^7 C3H-L 1/a tumour cells

Spleen cells transferred	Treatment‡ of recipients	Dead with tumour/total
Immune*	Nil	7/20†
Normal	Nil	16/16
Immune*	CPA 200 mg/kg	18/20
Normal	CPA 200 mg/kg	12/12

*Derived from C3H mice immunized twice by i.c. injection of 10^7 C3H-L 1/a cells.
†This group differ significantly at the 99% level from other groups (Fourfold Table Test).
‡Given the day before challenge.

From Table 4 it appears that the simultaneous i.p. injection of C3H-L 1/a cells and immune spleen cells is followed by an antibody production which is larger than the antibody production released by the injection of tumour cells or immune spleen cells alone, or by the simultaneous i.p. injection of tumour cells and normal spleen cells. Immunofluorescence indices above 0.3 were not seen until after the third day after the injection of cells.

Table 5 shows that protection against an i.p. graft of C3H-L 1/a tumour cells was afforded by simultaneous treatment of the mice with immune serum.

Even though the spleen responded to intraluminal caecal immunization, the spleen was not, as Table 6 shows, imperative for obtaining protection against the subsequent graft by i.c. immunization with ascites tumour cells. Nor was the spleen imperative for the antibody production following intracaecal immunization.

DISCUSSION

The study confirms that isograft resistance to malignant ascites cells could be obtained by a prior inoculation of the cells into the caecal lumen of C3H mice.

The immunizing cells were inoculated through a needle in laparotomized mice. A possible unspecific stimulation due to this pro-

*Table 4. Specific antibody production in C3H mice injected i.p. with C3H-L-ascites cells and spleen cells**

Recipients bled after	7 days			10 days		
Serum dilution	1:2	1:8	1:32	1:2	1:8	1:32
Transfer of	Fluorescent index (mean of two tests)					
Immune spleen cells†	0.15	0.15	0.17	0.13	0.25	0.17
Immune spleen cells† and ascites cells	1.00	0.97	0.86	0.82	0.96	0.92
Normal spleen cells and ascites cells	0.45	0.22	0.03	0.50	0.26	0.00
Ascites cells	0.48	0.18	0.05	0.73	0.29	0.06

The fluorescent indices were all below 0.30 when using sera obtained 3 days after transfer of spleen cells.
*Dose: 10^8 spleen cells, 2×10^6 C3H-L 1/a cells.
†The donors were immunized twice by the i.c. injection of 10^7 C3H-L 1/a cells at one week's interval.

cedure was ruled out by experiments with dose response. The protection obtained was correlated to the number of cells inoculated i.c. In groups receiving 10^5 and 10^6 cells i.c. through the wall of the caecum no protection was observed.

The immune response which occurred both intestinally and extraintestinally seemed to be cell-mediated as well as mediated by humoural factors.

The protection obtained, as described previously [6], could be transferred to untreated animals by immune spleen cells. But when the immune response of the recipients was inhibited by pretreatment with cyclophosphamide, transferrence of immune spleen cells was without effect.

This indicates that the effect of immune spleen cells did not depend on passive immunity exclusively. The temporary production of an ascites tumour, observed in some mice receiving immune spleens, supports this conclusion.

The *in vitro* test for cell-mediated immunity indicates that T-cells, at least in the intestinal wall and mesenteric lymph nodes, can be active in inhibition of tumour growth in the present model system.

The effect of cyclophosphamide may indicate that the protective effect of transferred immune spleen cells is partly based on a multistep reaction. Possibly other cells with immune competence may be activated by the transferred spleen cells.

Table 5. Adoptive immunization with serum

Recipients* treated with	Dead with tumour/group
Immune serum† ×1	5/16
Immune serum† ×3	0/17
Control serum ×3	20/20

Groups treated with immune serum differ according to the Fourfold Table Test significantly at the 99% level from control group.
*C3H mice grafted with 10^7 C3H-L 1/a tumour cells i.p. simultaneously with 0.5 ml serum. The same volume of serum was further given i.p. to two of the groups 2 and 4 days later. Survivors observed for 3 months.
†Donors twice immunized by inoculation of 10^7 C3H-L 1/a cells into the caecal lumen.

Table 6. Influence of splenectomy on the immune responses to ascites cells inoculated intracoecally (i.c.)

	Dead with tumour/group	Fluorescent index Serum dilution 1:8	1:16	1:32
Splenectomized and immunized	0/18	0.82	0.63	0.42
Sham-operated and immunized	0/10	0.88	0.72	0.38
Non-immunized	15/15			

C3H mice splenectomized or sham-operated. Two weeks later twice immunized by i.c. inoculation of 10^7 C3H-L 1/a tumour cells. After another 2 weeks the mice were bled and challenged with an i.p. graft of 10^7 C3H-L 1/a cells. Survivors observed for 3 months.

However, other cell subpopulations apart from T-lymphocytes are known to be effective against tumours. One is the group of cells described as K-cells which, even when taken from non-immunized animals, are able to lyse tumour cells coated with antibody [13]. The K-cells require very few specific antibodies to lyse tumour cells [13].

In opposition to the assumption of K-cell mediated immunity, however, it may be argued that small doses (10^5–10^6) of i.c.-injected C3H-L 1/a cells released a measurable antibody response but no protection against a secondary i.p. challenge. Furthermore, previous studies [6] failed to demonstrate any protective effect of 0.2-ml immune serum injected i.p. into the recipients together with the ascites tumour cells.

Another cell type with anti-tumour activity is the natural killer cell (NK-cell). Spleen cells from certain mouse strains kill without immunization a variety of murine tumour cells [14, 15].

In the present study spleen cells from non-immunized animals, when given simultaneously with a graft of the ascites tumour were without protective effect, indicating that NK-cells did not play a major role in the protection against C3H-L 1/a cells.

The effector mechanism in the present model system might have a humoural nature, too, presumably specific antibodies. This supposition is supported by the observation that 0.5-ml immune serum was able to protect most of the C3H mice against a simultaneously grafted ascites tumour. By repeated treatment with immune serum full protection against the graft was obtained. It is further supported by the finding that C3H-antiserum in presence of complement had cytotoxic activity towards C3H-L 1/a cells *in vitro* [9]. Moreover, the present study shows that in the presence of antigen the transferred spleen cells induced production of specific antibodies. The latency in the production indicates that active immunity of the recipients was needed.

The spleen responded to intraluminal injection of malignant ascites cells into the caecum, but the spleen was not imperative for the protection against a subsequent graft with these cells.

Acknowledgements—The excellent technical assistance of Marianne Barfoed is much appreciated. The Fibiger Laboratory is sponsored by The Danish Cancer Society.

REFERENCES

1. P. Brandtzaeg and K. Baklien, Immunohistochemical studies of the formation and epithelial transport of immunoglobulins in normal and diseased human intestinal mucosa. *Scand. J. Gastroent.* **11,** Suppl. 36 (1976).
2. Y. Levanon, H. Raettig and S. Rossetini, Positive immunological reaction of gut cells from orally immunized animals demonstrated by passive cutaneous anaphylaxis. *Experientia* **24,** 600 (1968).
3. P. A. Crabbe, D. R. Nash, H. Bazin, H. Eyssen and J. F. Heremans, Antibodies of the IgA type in the intestinal plasma cells of germfree mice after oral or parenteral immunization with ferritin. *J. exp. Med.* **130,** 723 (1969).
4. J. Dolozel and J. Bienenstock, IgA and non-IgA immune response after oral and parenteral immunization of the hamster. *Cell. Immunol.* **2,** 458 (1971).
5. H. J. C. Lund, Vaccination of rats against the Yoshida ascites sarcoma with the formation of complement-fixing antibody. *Brit. J. Cancer* **11,** 475 (1957).
6. M. L. Laursen and K. Laursen, Immunization by the intestinal route of C3H mice against C3H-L-ascites tumour cells. *Proc. Soc. exp. Biol. (N.Y.)* **154,** 314 (1977).
7. M. L. Laursen and K. Laursen, Enhancement of tumour growth in two syngeneic C3H-murine systems induced by immunization via the intracaecal route. *Brit. J. Cancer* **37,** 1039 (1978).
8. J. Kieler, C. Radzikowski, J. Moore and K. Ulrich, Tumorigenicity and isoimmunizing properties of C3H mouse cells undergoing "spontaneous" malignant conversion *in vitro*. *J. nat. Cancer Inst.* **48,** 393 (1972).
9. C. Radzikowski and J. Kieler, Serological studies of surface antigenic modification of C3H mouse cell lines undergoing "spontaneous" malignant conversion *in vitro*. *Arch. Immunol. Ther. exp. (Warsz.)* **20,** 461 (1972).
10. G. Möller, Fluorescent antibody technique for demonstration of isoantigens in mice. *Meth. med. Res.* **10,** 58 (1964).

11. E. KLEIN and G. KLEIN, Antigenic properties of lymphomas induced by the Moloney agent. *J. nat. Cancer Inst.* **32,** 547 (1964).
12. H. O. SJÖGREN, Blocking and unblocking of cell-mediated tumor immunity. *Meth. Cancer Res.* **10,** 19 (1973).
13. H. PERLMANN, P. PERLMANN, U. HELLSTRÖM and S. HAMMARSTRÖM, Purification and fractionation of human blood lymphocytes. Characterization of subclasses by their antibody-dependent cytotoxic potential (K-cell assay). In Vitro *Methods in Cell-Mediated and Tumor Immunity*. (Edited by B. R. Bloom and J. R. David) p. 497. Academic Press, New York (1976).
14. R. G. KIESSLING, E. KLEIN, H. PROSS and H. WIGZELL, Natural killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cells. *Europ. J. Immunol.* **5,** 117 (1975).
15. G. PETRANYI, R. G. KIESSLING and G. KLEIN, Genetic control of natural killer lymphocytes in the mouse. *Immunogenetics* **2,** 53 (1975).