PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 314-317 (1977)

Immunization by the Intestinal Route of C3H-Mice against C3H-L-Ascites Tumor Cells (39661)

M. LAU LAURSEN AND KIRSTEN LAURSEN
With the technical assistance of Marianne Barfoed

The Fibiger Laboratory, Ndr. Frihavnsgade 70, DK-2100 Copenhagen Ø, Denmark, and Medical Department Y, Bispebjerg Hospital, DK-2400 Copenhagen NV, Denmark

Introduction. In a report presented in 1880 to the Academie des Sciences in Paris, the idea of oral vaccination as a means of conferring immunity was suggested by Pasteur, Chamberland, and Roux (1). These eminent authors pointed out that chickens not only survived but were rendered immune after ingesting food saturated with the organism of chicken cholera. In 1891, Ehrlich (2) demonstrated antitoxin in the blood of mice immunized by feeding them on cakes containing vegetable poisons such as ricin and abrin. The presence of specific antibody activity in intestinal secretions was demonstrated in 1922 by Davies (3) who examined the feces of patients suffering from dysentery. In 1957, Lund (4) reported that rats treated with intracecal injections of Yoshida's ascites tumor cells developed complement-fixing antibodies and resistance to secondary challenge with this tumor. Intestinal vaccination with viruses and bacteria has since been confirmed in numerous studies. The participation of the intestinal lymphoid system in the natural defense against cancer is not clear. However, in view of the fact that the intestinal wall is one of the major sites of lymphocyte production, this question deserves more attention. Studies of this type may provide us with a better understanding of variation in the immunoresistance to cancer. The present investigation confirms the isoimmunizing properties of malignant syngeneic tumor cells inoculated into the lumen of cecum in inbred mice.

Materials and methods. Seven- to eight-week-old inbred mice of the C3H/Fib strain were used for this work. The animals were kept under conventional conditions with up to 10 mice in each cage. The malignant cell material was derived from cultures of C3H mouse lung fibroblasts which had undergone apparently spontaneous malignant conversion and transformation during propagation *in vitro* (5). From these cells an ascites tumor (C3H-L 1/a) was established which grew equally well *in vivo* and *in vitro*. On intraperitoneal inoculation, TD_{50} was 2×10^6 cells. On subcutaneous inoculation, small tumors were produced which regressed within 2–4 weeks. After a small laparotomy, 10^7 cells in a volume of 0.1 ml were inoculated into the cecal lumen of ether-anesthesized mice using injection needle 27 Gx1. After the injection, the cecum was washed in isotonic saline before replacement. Specific antibodies were demonstrated by the indirect immunofluorescence technique, using live C3H-L 1/a cells as target cells and serial dilutions of mouse sera obtained by bleeding from the retroorbital sinus. The sera were stored at −70°. Staining was done with fluorescein-conjugated pig anti-mouse globulin purchased from Hyland. The fluorescent index was calculated as described by Klein and Klein (6). Tumor neutralization tests were made with lymph node cells, splenocytes, and antisera obtained from donors immunized by intracecal inoculation of 10^7 C3H-L 1/a cells. Lymph nodes and spleens were removed under sterile precautions and single-cell suspensions were prepared in phosphatebuffered saline (PBS) after gentle homogenization of the lymphoid organs in a glass homogenizer with a loose fitting pestle. C3H-L 1/a tumor cells were added to the lymphocyte suspension at a lymphocyte/tumor cell ratio (L/T) of 7/1 or 15/1, and the tumorigenicity of the mixed cell population was finally tested by intraperitoneal injection into nonsensitized C3H recipients at a tumor cell dose of 10^7 per animal. In similar experiments C3H-L 1/a cells were suspended in undiluted antisera at a concentra-

ISSN 0037-9727

tion of 10^7 cells per 0.4 ml. No complement was added. Indirect immunofluorescence tests on these suspensions showed 100% reaction. The experiments were discontinued 100 days after challenge.

Results. The protective effect of lumenal intracecal (ic) inoculation of 10^7 malignant C3H-L 1/a cells against secondary intraperitoneal challenge 8–26 days later with the same malignant cells is shown in Table I. As can be seen, a total of 72 (92%) of the 78 immunized mice survived challenge with 10^7 C3H-L 1/a cells. The survival rate in the control group was only 24% (5/21). Table I also shows that neither frozen and thawed cells nor supernatant fluid from such a frozen and thawed cell suspension gave rise to a protective effect when inoculated ic against a secondary ip challenge with live cells. Table II shows the results of the indirect immunofluorescence tests carried out with pooled sera from eight mice inoculated once or twice with 10^7 live C3H-L 1/a cells ic. The animals were sacrified 9 days after the last ic inoculation. It appears that even at a dilution of 1:16, the fluorescent index was above 0.3. The indirect immunofluorescence test did not show new antibodies to membrane antigens on C3H-L 1/a cells in a sera from mice immunized ic with frozen and thawed cells. Neither did this technique give positive results when using sera from mice immunized ic or sc with supernatant fluid from frozen and thawed cell-suspension. Tumor neutralization tests were made with sera and lymphocytes from C3H mice immunized twice by the ic inoculation of 10^7 C3H-L 1/a cells 2 and 3 weeks before sacrifice. In animals reimmunized after 1 week, the cecum and the abdominal cavity were examined but no indication of tumors in or outside the cecum were seen. The results of three experiments are shown in Table III from which it appears that the antisera were without any tumor-neutralizing effect. Lymph node cells had no statistically significant effect, whereas splenocytes showed a pronounced inhibitory effect. In some of these experiments it was observed that the mice developed ascites the first 7 or 8 days, and to the same degree whether the splenocytes were transferred or not. After this period, however, the ascites suddenly disappeared in those groups which had received sensitized splenocytes mixed with the malignant cells. Table III also shows that preincubation of the tumor cells with specific antiserum for 30–60 min at 37° did not block the neutralizing effect of sensitized splenocytes.

TABLE I. PROTECTIVE EFFECT OF LUMENAL INTRACECAL (IC) INOCULATION OF MICE WITH 10^7 C3H-L 1/a CELLS AGAINST A SECONDARY CHALLENGE WITH THE SAME CELLS (IP) IN C3H/Fib MICE.

Days between ic and ip inoculations of live cells	Mice dead with tumor/total mice		
	Male	Female	Sum
8	1/10	0/8	1/18
15	0/10	2/10	2/20
26	0/10	1/10	1/20
26 and 18	2/10	0/10	2/20
Sum of immunized mice	3/40	3/38	6/78
Nonimmunized controls	8/11	8/10	16/21
Mice immunized ic with frozen and thawed cells × 5			8/9
Mice immunized ic with supernatant fluid from a frozen and thawed cell suspension			10/13

TABLE II. INDIRECT IMMUNOFLUORESCENCE TEST.[a]

Serum dilution	Fluorescent index[b]	
	Immunizing injection (number)	
	1	2
1:1	0.99	0.96
1:2	0.94	0.96
1:4	0.76	0.84
1:8	0.48	0.71
1:16	0.35	0.51
1:32	0.10	0.24

[a] Target cells: C3H-L 1/a. Sera: C3H mice inoculated once or twice ic with 10^7 C3H-L 1/a cells and bled 9 days after the last injection.

[b] (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 antiserum.

Discussion. By the ic inoculation of Yoshida's ascites tumor cells, Lund (4) induced immunity in rats against a secondary ip challenge with the same tumor cell line. This author also demonstrated specific complement fixing antibodies in ic immunized rats (4, 7). These experiments were carried out in an allogeneic system. In the present

TABLE III. TUMOR NEUTRALIZATION TEST.[a]

	Group							
	I		II[b]		III	IV	V	VI
	Sensitized lymph node cells		Sensitized splenocytes			Antiserum + sensitized splenocytes	Nonsensitized splenocytes	
Experiment	7:1[c]	15:1	7:1	15:1	Antiserum	15:1	15:1	Nihil
1	8/10[d]			1/10	7/8	0/10		9/10
	24 ± 3.0[e]			73	22 ± 2.2			23 ± 2.6
2	4/10	3/5	1/10	0/10	4/10	1/10		8/10
	24 ± 2.5	18 ± 3.0	23		25 ± 3.5	25		23 ± 2.6
3	5/6	11/17	4/18	2/23	16/19		14/18	17/20
	21 ± 3.8	23 ± 4.5	23 ± 1.8	20-2.0	20 ± 1.4		23 ± 2.4	23 ± 3.2
Sum of three experiments	17/26	14/22	5/28	3/43	27/37	1/20	14/18	34/40
(percentage dead)	65	64	18	7	73	5	78	85

[a] C3H/Fib-mice were immunized twice by ic inoculation of 10^7 C3H-L 1/a cells and used as donors. Serum, lymph node cells, and splenocytes from these donors were mixed with C3H-L 1/a cells, and the mixtures were transplanted ip to untreated C3H-Fib-mice. In one group (V) splenocytes from nonimmunized donors were used.

[b] Groups of II and IV significantly differ from the other groups at the 99% level.

[c] Lymphocyte/tumor cell ratio.

[d] Number of dead mice/total mice.

[e] Time of survival after challenge expressed in days.

study, syngeneic C3H-L 1/a tumor cells showed isoimmunizing properties in inbred C3H mice after ic inoculation of live malignant cells, but not after ic inoculation of frozen and thawed cells. The isoimmunizing effect was demonstrated by resistance to secondary ip challenge, the presence of specific antibodies as evidenced by a positive indirect immunofluorescence test, and by the protective effect of sensitized splenocytes in a tumor-neutralization test. The fact that frozen and thawed cells inoculated in the cecal lumen did not give rise to a protective effect against a later challenge indicates that the inoculated cells have to be viable and that macromolecules which are supposed to be liberated from the frozen cells are without any protective effect. Neither did Kieler *et al.* (5) obtain any evidence which supported the suspicion that antigenic properties of C3H-L 1/a-cells are due to virus.

From the observations made so far, it is not clear whether immune stimulation depends on the presence of intact tumor cells from tumor cells migrating into the intestinal epithelium or from tumor cells left in the intestinal wall at the site of injection.

Experiments to distinguish between these possibilities are now under investigation. It is also an open question whether the immunity is mediated by cells or antibodies. Cytotoxic antibodies have previously (8) been demonstrated in C3H mice hyperimmunized with C3H-L 1/a cells. However, these antibodies could only be demonstrated after 5–7 immunizing injections. The results of the present study indicate that the antibodies produced in ic immunized mice are without any protective or blocking effect in the tumor neutralization test. Experiments by Süssdorf (9) and Nieuwenhuis (10) indicate that the appendix is essential for the antibody-forming potential in the spleen, and Clancy and Bienenstock (11) stated that lymphocytes with surface IgG and IgM receptors from the Peyers patches especially seed to the spleen. This might explain the pronounced effect of splenocytes and the insignificant effect of lymph node cells observed in our experiments, provided that the tumor-neutralizing effect of sensitized splenocytes depends on the transfer of activated B cells. The temporary production of an ascites tumor which regressed after 7–8 days suggests that protection did not depend on passive immunity exclusively. Experiments aimed at answering this question are in progress.

Summary. Lumenal intracecal inoculation of viable malignant cells produces a protective effect against a secondary ip challenge with the same tumor cell. Cells inoculated in the lumen of the cecum showed immunizing properties as demonstrated by specific antibodies evidenced by a positive indirect immunofluorescence test.

Furthermore, the protective effect can be

transferred to an untreated animal by sensitized splenocytes.

This work was supported by the Danish Cancer Society.

1. Pasteur, L. (1880) cited by Calmette, A., Ann. Inst. Pasteur **37,** 900 (1923).
2. Ehrlich, P., Deuth. Med. Wochenschr. **17,** 976, 1218 (1891).
3. Davies, A., Lancet **203,** 1009 (1922).
4. Lund, H., Brit. J. Cancer **11,** 475 (1957).
5. Kieler, J. et al., J. Nat. Cancer Inst. **48,** 393 (1972).
6. Klein, E., and Klein, G., J. Nat. Cancer Inst. **32,** 547 (1964).
7. Lund, H., Acta Pathol. Microbiol. Scand. **43,** 391 (1958).
8. Radzikowski, C., and Kieler, J., Arch. Immunol. Ther. Exp. **20,** 461 (1972).
9. Süssdorf, D. H., J. Inf. Dis. **105,** 238 (1959).
10. Nieuwenhuis, P., "On the Origin and Fate of Immunologically Competent Cells," 162 pp. Wolters-Noordhoff, Groningen (1971).
11. Clancy, L. R., and Bienenstock, J., *in* "Immunological Aspects of the Liver and Gastrointestinal Tract" (Anne Ferguson and R. N. M. MacSween, eds.), p. 124. MTP Press, Lancaster, England (1976).

Received July 26, 1976. P.S.E.B.M. 1977, Vol. 154.