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Enhanced Immunogenicity of Chemically-Coated Syngeneic Tumor Cells

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ABSTRACT The immunogenicity of the EL-4 strain of mouse lymphoid leukemia cells in syngeneic C57BL/6 recipients was enhanced by *in vitro* coating of the cells with either Concanavalin A or dinitrophenylaminocaproate. The tumor-specific immune response was quantitated using a recently developed assay that involves *in vitro* cell-mediated cytotoxicity.

Tumor-specific transplantation antigens have been demonstrated on many tumors. These antigens, however, are generally weak immunogens, especially when the tumor cells are injected into syngeneic recipients (1). Attachment of foreign antigens to the tumor-cell surface has been suggested as a means of enhancing the immunogenicity of tumor antigens (1). We have compared the immunogenicity in syngeneic C57BL/6 mice of unmodified mouse lymphoid leukemia cells (EL-4) with that of tumor cells coated *in vitro* with either concanavalin A (Con A; ref. 2) or 2,4-dinitrophenylaminocaproate (DnpC). Immunogenicity of tumor cells was indicated by the appearance in the spleens of recipient mice of cytotoxic "effector" cells capable of causing the release of ^{51}Cr *in vitro* from labeled tumor cells.

MATERIALS AND METHODS

Tumors

Two lymphoid-tumor cell lines were used in this study (*i*) EL-4, originally induced in C57BL mice by the carcinogen 9:10-dimethyl-1:2-benzanthracene, and since carried in several laboratories as a transplantable ascites tumor (3), and (*ii*) LSTRA, an ascites tumor induced in BALB/c mice. The tumors have been maintained in our laboratory by twice-weekly intraperitoneal passage in C57BL/6 and BALB/c mice, respectively. All mice were obtained from the Animal Production Unit of the National Institutes of Health.

Materials

Twice-crystallized Con A was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. The *N*-hydroxysuccinimide ester of 6-(2,4-dinitrophenyl)aminocaproic acid (DnpC-ester) was prepared by reacting a mixture of 6-(2,4-dinitrophenyl) aminocaproic acid and *N*-hydroxysuccinimide (0.1 M each) in 1,2-dimethoxyethane with a 10% molar excess of dicyclohexylcarbodiimide. The reaction proceeded for 16 hr at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in 1,2-dichloroethane and extracted with 0.2 M $NaHCO_3$. The lower phase was dried with anhydrous Na_2SO_4. The active ester was recovered as a crystalline precipitate after adding an equal volume of hexane at 65°C and allowing the mixture to cool. The preparation was dried under reduced pressure. Samples of DnpC were dissolved in dioxane at a concentration of 5 mg/ml prior to use. Dinitrofluorobenzene (FDNB) was purchased from Eastman Organic Chemicals, Rochester, N.Y. The media used were provided by the Media Section, NIH.

Chemical coating of tumor cells

EL-4 cells were harvested from the peritoneal cavity 5 days after the intraperitoneal inoculation of mice with 10^6 tumor cells. The medium used was bicarbonate-free Hanks' Balanced Salt Solution. The cells were given 1500 R irradiation and were then subjected to one of the following treatments. (*i*) nil; (*ii*) incubation, at a concentration of 10^7 cells per ml, with 25 μg/ml of Con A for 30 min at 37°C, or (*iii*) reaction with 100 μg of DnpC-ester at room temperature for 10–15 sec at a cell concentration of 5×10^7 cells/ml. After these treatments, 50 ml of ice-cold medium was added to the tubes and the cells were centrifuged and washed once. Cells exposed to DnpC-ester acquired a marked yellow color.

Immunization

Mice were given two intraperitoneal injections of 5×10^5 and 5×10^7 cells, respectively, at either a 3- or 4-week interval. 10 days prior to receiving their first injection of Con A-coated EL-4 cells, some mice were injected intraperitoneally with either 10 or 100 μg of Con A in saline. Similarly, 10 days prior to receiving DnpC-coated tumor cells, a group of mice was skin painted with DNFB as described by Iverson (4).

In vitro cytoxicity assay for cell-mediated immunity

This assay is described in detail elsewhere (5). 10 days after their second injection of tumor cells, mice were killed and their spleens were removed. 5×10^6 spleen cells, suspended in Eagle's Basal Medium supplemented with 10% heat-inactivated fetal bovine serum and 200 μg/ml of the complement inactivator, carrageenan, were added to 5×10^4 target cells (either EL-4, LSTRA, or normal C57BL/6 lymph node cells) labeled with ^{51}Cr. After incubation on a rocking platform at 37°C for 4 hr, the cells were harvested, centrifuged, and the radioactivity of the supernatant fluid was measured. A sample of labeled target cells was repeatedly frozen and thawed so that the maximum possible ^{51}Cr release could be ascertained. The percentage of the maximum ^{51}Cr release from both target cells exposed to normal spleen cells and to immune spleen cells

Abbreviations: Con A, concanavalin A; DnpC, 6-(2,4-dinitrophenyl)aminocaproate; DnpC-ester, *N*-hydroxysuccinimide ester of 6-(2,4-dinitrophenyl)aminocaproic acid; FDNB, 2,4-dinitrofluorobenzene.

TABLE 1. *Uptake of ^{125}I-labeled concanavalin A*

Cell type	Con A bound per 10^7 cells (ng)	Average no. of molecules bound per cell
Lymph-node cells	51.6	4.38×10^4
EL-4 cells	233.0	19.80×10^4

was measured. The specific release of ^{51}Cr by the immune spleen cells was obtained by subtracting the percentage released from target cells exposed to normal spleen cells from that released by target cells exposed to immune spleen cells.

Statistical analysis

Each spleen-cell sample was tested in quadruplicate. The mean percentage release by the normal spleen cells was used to calculate four individual determinations of the specific release by the immune spleen cells. The mean and standard error (S.E.) of the percentage of specific ^{51}Cr release were calculated. The S.E. was approximated by the formula:

$$\frac{1}{FT}\left[\left(\frac{\text{Variance}}{n}\right)_{\text{i.s.}} + \left(\frac{\text{Variance}}{n}\right)_{\text{n.s.}}\right]^{1/2}$$

where FT is maximum (freeze-thaw) ^{51}Cr release, n is sample number, i.s. is immune spleen and n.s. is normal spleen. Student's "t" test for small samples was used to evaluate the significance of the difference between the ^{51}Cr release with immune spleen cells and normal spleen cells. Variances were not assumed to be equal. A P value of <0.01 was regarded as being significant.

***In vitro* cytotoxicity assay for humoral immunity**

Blood was collected from immunized mice and the sera tested for cytotoxicity against ^{51}Cr-labeled EL-4 cells in the presence of rabbit complement (6).

Uptake and toxicity studies of Con A and DnpC

EL-4 cells were reacted with 25 μg/ml of Con A containing a trace amount of ^{125}I-labeled Con A. (The Con A was kindly radioiodinated by Dr. W.E. Paul, NIH.) After the incubation procedure described above, the residual radioactivity of the cells was measured. From this value, the amount of Con A bound per 10^7 cells was calculated. The molecular weight of Con A is known to be 71,000 (2). Using this value we determined the average number of molecules of Con A bound per cell.

^{51}Cr-labeled EL-4 cells were incubated at 37°C for 30 min, either alone or in the presence of 25 μg/ml of Con A. At the end of the incubation period, an aliquot of uncoated cells was exposed to DnpC-ester for 15 sec. The uncoated, DnpC-coated, and Con A-coated cells were centrifuged and the amount of ^{51}Cr in each supernatant was measured. The cell pellets were resuspended in a 0.5-ml volume of medium and left at 4°C for 1 hr. The cells were again centrifuged and the amounts of ^{51}Cr released during the period measured.

RESULTS

Uptake and toxicity of Con A and DnpC for EL-4 cells

The amount of Con A bound to, or incorporated into, EL-4 cells after *in vitro* incubation with 25 μg/ml of ^{125}I-labeled Con A was calculated. The comparative uptake by normal lymph node cells was also determined. As shown in Table 1, EL-4 tumor cells bound significantly more Con A per cell than did normal lymphocytes.

^{51}Cr-labeled EL-4 cells were exposed to medium alone, Con A, and DnpC-ester. The cells were washed and left at 4°C for 1 hr. The amounts of ^{51}Cr released from the cells during both the coating procedure itself and the hour at 4°C were measured. These amounts were expressed as percentages of the release obtained by freezing and thawing an equal number of labeled cells, Table 2. Exposure to either Con A or DnpC-ester resulted in no apparent immediate or delayed cytotoxicity of EL-4 cells.

Immunogenicity of chemically-coated EL-4 cells

C57BL/6 mice were injected twice, intraperitoneally, with irradiated but otherwise untreated EL-4 cells or with irradiated cells coated with Con A or DnpC. The second injection was given either 3 weeks (Experiment *A*) or 4 weeks (Experiment *B*) after the first. The mice were killed 10 days after the second injection and their spleen cells were assayed for their ability to induce specific immune lysis of ^{51}Cr-labeled EL-4, C57BL/6 lymph node, and LSTRA cells. The results of Experiments *A* and *B* are shown in Table 3. Only one of eight mice immunized with untreated EL-4 cells showed slight activity; the others were completely inactive. By contrast, 7 of 8 mice immunized with Con A-coated EL-4 and 4 of 8 mice immunized with DnpC-coated cells showed significant immunity. Mice immunized with 10 μg of Con A prior to receiving Con A-coated cells showed activity in 4 of 5 animals tested, while those immunized with 100 μg of Con A showed slight activity in only 1 of 5 mice tested.

The mice in Experiment *B* were bled before death and their sera were tested for cytotoxic anti-EL-4 antibody. No antibody was detected in any of the sera.

Specificity of immune response in mice immunized with DnpC-coated EL-4

DnpC-coated EL-4 cells were injected twice into eight C57BL/6 mice, four of whom had been previously skin painted with FDNB, and into four BALB/c mice. 10 days after the second injection, their spleens were removed and assayed for activity against uncoated and DnpC-coated EL-4 and LSTRA cells. The percentages of specific immune lysis for the pooled spleen cell suspensions from each group are shown in Table 4. Antitumor activity was again noted in C57BL/6 mice injected with DnpC-coated EL-4 cells. This activity appeared to be specific for EL-4 antigens rather than DnpC since no significant lysis was induced in DnpC-coated LSTRA cells. Similarly, BALB/c mice immunized with DnpC-coated EL-4 cells showed only a slight reaction against DnpC-coated LSTRA cells, and achieved a similar percentage lysis against uncoated and DnpC-coated EL-4 cells.

TABLE 2. *Percentage ^{51}Cr release from labeled EL-4 cells exposed to Con A and DnpC*

Coating of cells	% Release after incubation at 37°C	Additional release after 1-hr incubation at 4°C
Nil	6.6	3.2
Con A	6.9	1.8
DnpC	7.3	3.0

TABLE 3. *In vitro cell-mediated cytotoxicity of spleen cells from C57BL/6 mice immunized with EL-4 tumor cells*

Immunogen	Expt.	Percentage lysis* ± SE: EL-4 (C57BL/6)	Lymph node (C57BL/6)	LSTRA (BALB/c)
EL-4 untreated	*A*	−0.71 ± 0.36	−0.17 ± 0.67	−2.89 ± 0.89
		−1.40 ± 0.22	−2.11 ± 1.41	−1.03 ± 0.87
		−0.97 ± 0.24	0.50 ± 0.52	1.06 ± 1.47
		−0.95 ± 0.23	−1.28 ± 0.99	0.00 ± 1.29
	B	−0.28 ± 0.31	−0.50 ± 1.50	N.T.‡
		−0.27 ± 0.34	0.26 ± 1.16	N.T.
		−0.66 ± 0.32	0.30 ± 1.32	N.T.
		†1.38 ± 0.34	−0.83 ± 1.70	N.T.
DnpC-coated EL-4	*A*	†4.29 ± 0.25	−0.74 ± 1.40	−0.49 ± 1.67
		†5.58 ± 0.28	−0.65 ± 1.43	−0.98 ± 1.98
		0.19 ± 0.27	−0.13 ± 1.40	−2.20 ± 1.87
		−0.50 ± 0.35	−2.28 ± 1.95	−1.58 ± 1.65
	B	−0.02 ± 0.21	−0.81 ± 1.56	N.T.
		0.01 ± 0.25	0.34 ± 1.33	N.T.
		†4.79 ± 0.32	−0.02 ± 1.61	N.T.
		†4.60 ± 0.25	1.23 ± 1.29	N.T.
Con A-coated EL-4	*A*	†6.00 ± 0.55	−1.52 ± 1.42	−1.06 ± 1.83
		†5.24 ± 0.44	−0.21 ± 1.41	2.12 ± 1.70
		†2.31 ± 0.37	−1.80 ± 1.51	0.03 ± 1.87
		†5.75 ± 0.26	−0.48 ± 1.63	−0.85 ± 1.60
	B	0.06 ± 0.30	1.08 ± 1.56	N.T.
		†3.80 ± 0.30	0.65 ± 0.86	N.T.
		†3.82 ± 0.22	0.82 ± 1.64	N.T.
		†4.62 ± 0.46	0.84 ± 1.56	N.T.
Con A-coated EL-4 in mice pretreated with 10 μg of Con A	*A*	†4.30 ± 0.34	−1.74 ± 0.87	−0.23 ± 1.30
		†4.69 ± 0.43	−2.22 ± 1.04	2.38 ± 1.27
	B	†6.38 ± 0.54	−1.20 ± 1.10	N.T.
		†1.94 ± 0.32	1.63 ± 0.81	N.T.
		†3.73 ± 0.32	0.30 ± 1.09	N.T.
Con A-coated EL-4 in mice pretreated with 100 μg of Con A	*A*	−0.24 ± 0.21	−1.85 ± 1.74	1.47 ± 1.77
		−0.45 ± 0.14	−0.85 ± 0.96	0.78 ± 1.17
	B	0.23 ± 0.26	1.34 ± 1.07	N.T.
		−0.11 ± 0.37	−1.28 ± 1.08	N.T.
		†1.70 ± 0.24	−0.08 ± 1.00	N.T.

* Target cells incubated with normal spleen cells resulted in the following percentage lysis (expressed as percentage of maximum releasable ^{51}Cr): EL-4: Expt. *A*, 10.09; Expt. *B*, 8.14; Lymph-node cells: Expt. *A*, 24.39; Expt. *B*, 31.03; LSTRA: Expt. *A*, 34.96. The percent lysis figures in the table represent the difference between percent lysis with immune cells versus normal cells. Target cells incubated with normal spleen cells do not release significantly more ^{51}Cr than target cells incubated in medium alone. Each value represents the mean of 4 determinations.

† Significant immune lysis: $P < 0.01$ by Student's "t" test.

‡ N.T. = Not tested.

TABLE 4. *In vitro cell-mediated cytotoxicity of spleen cells from mice immunized with DnpC-coated EL-4 tumor cells*

Recipient mice	Percentage lysis* ± SE: EL-4	DnpC-coated EL-4	LSTRA	DnpC-coated LSTRA
C57BL/6	4.38 ± 0.13	5.81 ± 0.56	−1.22 ± 0.55	0.61 ± 0.59
C57BL/6 skin painted with FDNB	0.50 ± 0.31	−1.09 ± 0.34	N.T.†	N.T.
BALB/c	36.36 ± 2.36	40.17 ± 1.66	0.69 ± 0.32	1.78 ± 0.79‡

* Target cells incubated with normal C57BL/6 spleen cells resulted in the following percentage lysis (expressed as percentage of maximum-releasable ^{51}Cr): EL-4, 6.29; EL-4–DnpC, 7.96; LSTRA, 9.54; LSTRA–DnpC, 9.50; target cells incubated with normal BALB/c spleen cells: EL-4, 7.10; EL-4–DnpC, 8.94; LSTRA, 6.79; LSTRA–DnpC, 8.78. Target cells incubated with normal spleen cells do not release significantly more ^{51}Cr than target cells incubated in medium alone. Each value represents the mean of 6 determinations.

† N.T. = Not tested.

‡ $0.05 > P > 0.01$.

Included in the above experiment were C57BL/6 mice skin painted with FDNB prior to receiving DnpC-coated EL-4 cells. No anti-EL-4 immune response was noted in these mice.

DISCUSSION

Previously reported attempts to elicit tumor-specific immunity against EL-4 cells in C57BL/6 mice have been unsuccessful (7). Similarly, in our experiments, all but 1 of 8 mice showed no detectable immunity after injection of irradiated, but otherwise unaltered, EL-4 cells. By contrast, however, inoculation of irradiated and either Con A- or DnpC-coated cells elicited tumor-specific immunity in 7 of 8 and 4 of 8 mice, respectively. Thus, coating of the EL-4 tumor cells with these substances enhanced their immunogenicity.*

The ability of Con A to enhance tumor immunogenicity might account for the recent report of Shoham and his coworkers (8) that *in situ* injection of Con A into polyoma-induced tumors led to the regression of the tumors.

Our findings also support an earlier observation by Czajkowski and his colleagues (9) that human gammaglobulin coupled to mouse sarcoma cells rendered these cells immunogenic in isogeneic hosts. These authors suggested that the human gammaglobulin acted as an antigenic "carrier" molecule for the otherwise nonimmunogenic hapten-like tumor-specific transplantation antigen. This same principle was recently expounded by Mitchison (1), who suggested that dinitrophenyl groups attached to tumor cells might also provide suitable carrier molecules for tumor antigens. Our results show that this treatment does enhance immunogenicity, yet we believe that the mechanism remains obscure. Thus, the proposition that the DnpC and Con A acted as antigenic "carrier" determinants was not supported by the observation that skin painting with FDNB or injection of 100 μg of Con A prior to immunization with DnpC- and Con A-coated cells reduced the tumor-specific immunogenicity of these cells. Also, when spleen cells from mice successfully immunized with DnpC-coated EL-4 cells were tested for activity against DnpC-coated LSTRA cells, minimal ($0.05 > P > 0.01$) cell lysis occurred.

Experiments are in progress to test other possible mechanisms of the enhanced immunogenicity of chemically-coated tumor cells. An understanding of this mechanism, together with more effective chemical coating procedures, might enable the development of a stronger anti-tumor immunity than reported in this paper. Our preliminary results are encouraging and they clearly demonstrate the value of a sensitive *in vitro* assay of cell-mediated immunity in the development of successful approaches to cancer immunotherapy.

* The nature of the reactive antigen on EL-4 is uncertain. Although EL-4 is carcinogen induced, infectious leukemogenic virus is also present in our EL-4 cell line. (Kindly tested for by Drs. J. Hartley and W. Rowe, NIH.) The possibility therefore exists that the tumor-specific immune response is directed against a virus-induced cell-surface antigen rather than a carcinogen-induced cellular antigen.

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