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Immunosensitivity and Histocompatibility Antigens in Drug-altered Leukemic Cells[1]

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SUMMARY

New antigenic properties of experimental lymphomas have been reported previously following *in vivo* treatment with antitumor agents. 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) induced new antigenic characteristics on L1210 and L5178Y lymphomas, that were previously investigated in studies in animals compatible with the original untreated parental tumors. Here the L1210/DIC and L5178Y/DIC susceptibility to the cytotoxic effects of allogeneic and xenogeneic lymphocytes and sera obtained from animals sensitized to DBA/2 histocompatibility antigens were studied. The original and the DIC tumors showed the same sensitivity to anti-DBA/2 cellular and humoral cytotoxicity.

The immune response elecited in allogeneic mice by the original and DIC sublines was evaluated by *in vitro* cell-mediated and humoral cytotoxic assay. Beyond the immune response to histocompatibility antigens, a specific, anti-DIC-antigen immunoreaction was not found. Inhibition assay of the cell-mediated cytotoxicity and absorption of the humoral cytotoxicity demonstrated that DIC-induced antigens are not reciprocally related in cell-surface concentration to the natural DBA/2 histocompatibility antigens associated with tumor cells of DIC lines. An experiment was conducted in which specific activity against the DIC-treated L5178Y/DIC cells was observed with anti-L5178Y/DIC rabbit immune serum absorbed with the parental L5178Y lymphoma. This finding provides additional support to previous studies indicating that treatment with DIC induced new antigens on the lymphoma cells.

INTRODUCTION

It was previously demonstrated that immunogenic characteristics of mouse leukemic cells can be altered following *in vivo* drug treatment (1, 7, 11). As the new immunological properties were observed, even after the leukemic sublines were passaged weekly in immunosuppressed mice for more than 2 years without any further treatment, the hypothesis was advanced that the antigenic expression may have a genetic basis (1, 10). In some instances, the drug-modified tumor cells were markedly immunogenic and a heavy challenge of viable cells was rejected by mice compatible with the original unaltered tumor. Furthermore, specific susceptibility to *in vitro* cell-mediated cytotoxicity exerted by lymphocytes (9) and to complement-dependent humoral cytotoxicity (A. Nicolin, unpublished data) by sera of previously sensitized mice was observed. These findings and their interest for the immunotherapy of experimental tumors led to further investigations of the drug-altered leukemic cells.

In general, immunodepressed mice bearing an immunologically altered tumor show a greater median survival time than mice bearing the parental tumor (3, 6, 13, 14). To account for this increase in median survival time 3 hypotheses have been advanced: (*a*) increased immunosensitivity of the drug-treated sublines; (*b*) decreased oncogenicity of the altered cells; and (*c*) residual host-immune response to the antigens associated with the drug-treated tumors.

Moreover, both the hypotheses of increased immunosensitivity and decreased oncogenicity have received experimental support for some tumor sublines that had become drug resistant following chemotherapeutic treatment (5, 14, 16).

The immunosensitivity to cellular and humoral cytolytic effects of 2 leukemic sublines, previously shown to be immunologically altered by treatment with DIC,[3] has been evaluated in the current study. In addition, the integrity of histocompatibility antigens in the drug-treated cells has been examined. The neoplastic transformation may be associated, in most instances, with the appearance of tumor-specific transplantation antigens, and some studies have indicated a reciprocal relationship between the histocompatibility specificities and tumor-specific transplantation antigens associated with the neoplastic transformation (4, 15). Similarly, the drug-induced antigens might provoke a loss of histocompatibility antigens. The total amount of histocompatibility specificities was evaluated in the present study by the inhibition assay of cell-mediated cytotoxicity and by absorption assay of cytotoxic antibodies. The inhibition exerted by L1210/DIC, L5178Y/DIC leukemic sublines, and original lymphomas on immune lymphocytes and on antisera activity against histocompatibility antigens has been investigated.

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[3] The abbreviation used is: DIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide.

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MATERIALS AND METHODS

Animals. Inbred DBA/2 Cr(H-2^d), C57BL/10 ScSnCr(H-2^b), C3H Cr(H-2^k), and BALB/c Cr × DBA/2 Cr F_1 ($CD2F_1$) mice of both sexes weighing 20 to 25 g were used. Outbred Sprague-Dawley rats and New Zealand White rabbits were also used in this study.

Tumors. L1210 leukemia and L5178Y lymphoma were maintained by weekly passages i.p. in compatible $CD2F_1$ mice. L1210/DIC and L5178Y/DIC were developed following 7 to 10 i.p. daily treatments *in vivo* of L1210 and L5178Y with DIC (100 mg/kg i.p.) and serial passage as previously reported (1) and maintained in immunosuppressed (cyclophosphamide, 200 mg/kg i.p. 24 hr before tumor challenge) $CD2F_1$ mice.

Effector Cells. Immune lymphocytes against DBA/2 histocompatibility antigens were obtained utilizing spleens of allogeneic mice previously sensitized (10 days before) by s.c. grafting of DBA/2 skin (10 pieces, 1 cu mm) and a 10^8 i.p. inoculum of DBA/2 spleen cells. Rat immune lymphocytes were obtained in the same manner by grafting skin (30 pieces) and injecting i.p. 10^9 spleen cells.

Lymphocytes immune against tumor were obtained utilizing mouse spleens of C3H or C57BL/10 mice inoculated i.p., 10 days earlier, with 10^7 viable leukemic cells. Lymphocyte and tumor cell viability was checked by the dye exclusion test.

Immune Sera. Sera against normal DBA/2 tissues and against the 4 tumors were obtained, as described for immune lymphocytes, but using multiple immunizations at 2-week intervals.

For preliminary evaluation of the activity, blood from mice was obtained from the retroorbital sinus. After further stimulation, sera from mice, rats, or rabbits were collected, pooled, inactivated (56° for 30 min), and stored at −70° in 1-ml vials.

Cytotoxicity Assay. The ^{51}Cr release test by labeled target cells was used for cytotoxicity assay, as described by Wigzell (17) and modified by Canty (2).

In each experiment the activity of effector cells or immune sera was compared against the DIC cells and the corresponding unaltered cells.

Inhibition Assays of Cellular Cytotoxicity. Washed spleen cells (10^7) (effectors) from normal (control) or immune mice, suspended in a plastic Petri dish in 1 ml Medium 199 supplemented with 10% heat-inactivated fetal calf serum, were incubated on a rocking platform with 5×10^4 (in 50 μl) ^{51}Cr-labeled tumor cells (targets) at 37° in a moist atmosphere of 10% CO_2:90% air for 4 hr. The inhibition assay was conducted as described by Ortiz de Landazury (12) by concomitant addition of unlabeled cells (2×10^5 in 50 μl) to the suspension of effector cells:labeled target cells.

Humoral Cytotoxicity. ^{51}Cr-labeled target cells (10^5 in 100 μl) were incubated in plastic test tubes (60 × 11 mm) for 1 hr at 37° with 100 μl of serial 2-fold dilutions of the serum and with 100 μl of a 1:5 diluted guinea pig serum as complement source.

Serum absorption was carried out by classical methods. The cytotoxic effect of complement alone or serum alone was also checked, and the ^{51}Cr release was never more than 2% higher than that observed with labeled target cells in Medium 199 supplemented with 10% heat-inactivated fetal calf serum.

Spontaneous release in the 4-hr assay (for cellular cytotoxicity) was 15 ± 3%, and it was 10 ± 2% in the 1 hr assay (for humoral cytotoxicity).

The percentage of cytotoxicity (quadruplicate samples for each experimental group) was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm in experimental samples} - \text{cpm in controls}}{\text{cpm in 4x frozen-thawed samples} - \text{cpm in controls}} \times 100$$

RESULTS

The susceptibility to cell-mediated lysis was determined for L1210 and L5178Y tumors of DBA/2 origin and for the L1210/DIC and L5178Y/DIC sublines. Immune spleen cells of C3H(H-2^k) and C57BL/10(H-2^b) mice and rats previously sensitized to DBA/2(H-2^d) normal tissues, L1210 and L5178Y leukemias, and their DIC-treated sublines, were used. No quantitative differences in the lytic activity against the original parental and the transformed cells were exerted by allogeneic or xenogeneic immune lymphocytes (Table 1).

The susceptibility of the 2 original and DIC-treated tumor lines to C3H cytotoxic serum immune against DBA/2 normal tissues was determined. As may be seen in Chart 1, the complement-mediated serum cytotoxicity test resulted in the same percentage of ^{51}Cr release in parental and DIC-treated cells. Similar results were obtained using anti-DBA/2 sera from C57BL/10 mice and from outbred rats. Thus, the original and DIC-treated sublines did not reveal any substantial differences in their susceptibility to the humoral as well as cell-mediated, anti-DBA/2 cytotoxicity.

Sera immune to L1210, L5178Y, and to DIC sublines were obtained in C3H mice, and the lytic activity was determined against the tumor cells. Again the cytotoxic sera exerted the same activity against the original unaltered and the DIC cells (Chart 2).

These experiments, similar to those measuring cell-mediated cytotoxicity, resulted in the failure, by allogeneic or xenogeneic direct cytotoxic assay, to distinguish the original cells from the DIC cells.

An evaluation of alloantigens detectable on the original unaltered and DIC cell surface was carried out by the inhibition assay of cell-mediated cytotoxicity and by the absorption of humoral cytotoxic activity.

C3H lymphocytes, immune to DBA/2 normal tissues or to L1210 and L5178Y leukemias or DIC-treated sublines, were incubated with ^{51}Cr-labeled tumor cells along with unlabeled tumor cells. The inhibition of ^{51}Cr release exerted by cold cells is reported in Table 2. As the same inhibitory activity was produced by addition of nonlabeled original parental and DIC cells, it would appear that the tumor cells carry comparable amounts of alloantigens recognized by C3H mice. These data were confirmed further by the inhibition assay involving activity of C57BL/10 lymphocytes sensitized to DBA/2 normal tissues (Table 2).

Sera obtained in C3H or C57BL/10 mice immune to DBA/2

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Table 1
Cytotoxic effect of C3H or C57BL/10 mouse or rat spleen cells immune against normal DBA/2 tissues, original parental or DIC-treated lymphoma lines

Effector spleen cells[a]		Target cells (% ^{51}Cr release)[b]			
Donor strain	Immune to	L1210	L1210/DIC	L5178Y	L5178Y/DIC
C3H	DBA/2[c]	18 ± 0.5[d]	16 ± 0.5	14 ± 0.2	13 ± 0.2
	L1210	52 ± 1.7	56 ± 0.4	34 ± 0.4	31 ± 2.2
	L1210/DIC	56 ± 1.1	56 ± 0.2	51 ± 1.5	52 ± 1.3
	L5178Y	55 ± 0.6	54 ± 0.5	45 ± 1.2	43 ± 0.9
	L5178Y/DIC	52 ± 0.7	55 ± 0.6	51 ± 0.7	53 ± 0.5
C57BL/10	DBA/2	12 ± 0.6	12 ± 0.4	14 ± 0.7	13 ± 0.3
	L1210	60 ± 1.9	57 ± 1.3	NT[e]	NT
	L1210/DIC	28 ± 0.5	30 ± 1.3	NT	NT
Rat	DBA/2	34 ± 1.5	37 ± 1	33 ± 0.9	32 ± 0.4

[a] 10^7 cells.
[b] 5×10^4 cells.
[c] DBA/2 normal tissues (skin and spleen cells).
[d] Mean ± S.E.
[e] NT, not tested.

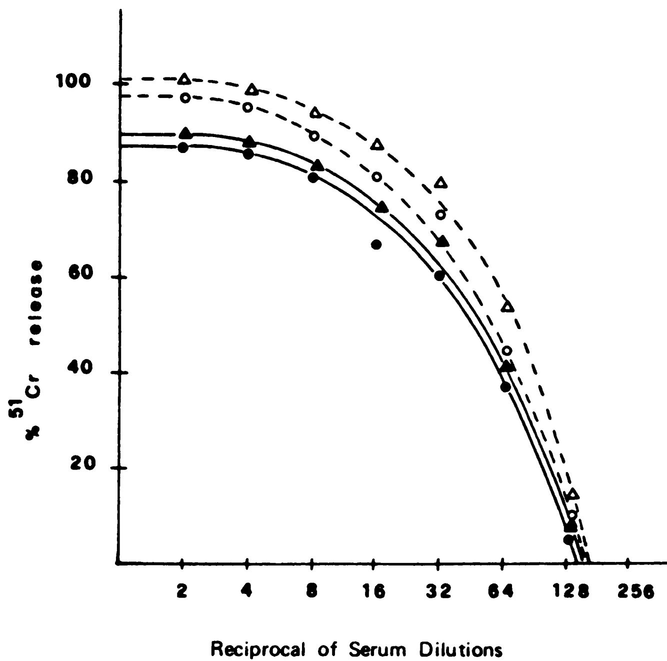

Chart 1. Cytotoxic activity of C3H antiserum immune against DBA/2 normal tissue. ●, L1210; ▲, L1210/DIC; ○, L5178Y; △, L5178Y/DIC. In all serological assays S. E. range was between 0.1 and 0.5.

normal tissues or to L1210 and L5178Y leukemic or DIC-treated sublines were absorbed with leukemic cells. The same residual activity was found in sera absorbed with the 2 parental and their corresponding leukemic lines (Table 3). Thus, the serum absorption assay did not show any gross differences in the amount of alloantigens (recognized by C3H and C57BL/10 strains) associated with the initial and DIC-treated cells.

Anti-L5178Y/DIC rabbit serum, after dilutions no longer lytic for original parental cells, showed cytotoxic activity against L5178Y/DIC cells (Chart 3).

Specific anti-L5178Y/DIC cytotoxicity was observed in L5178Y-absorbed rabbit serum immune to L5178Y/DIC cells (Chart 4*A*). In addition, unlabeled L5178Y or L5178Y/DIC cells were added to the suspension of ^{51}Cr-labeled L5178Y/DIC cells in anti-L5178Y/DIC serum that had been absorbed with L5178Y cells. The anti-L5178Y/DIC activity was specifically inhibited by addition of cold L5178Y/DIC cells. The activity of the immune serum was not lost on addition of cold L5178Y cells (Chart 4*B*).

DISCUSSION

In vitro and *in vivo* studies have indicated new antigenic properties of experimental tumor cells following *in vivo* treatment with drugs. However, a mildly increased median survival time of immunosuppressed mice bearing the modified tumors could suggest that factors, other than antigenic properties, might be involved in the alteration of drug-treated cells. On the basis of the increased host survival, it was considered that DIC-altered tumor cells, such as L1210/DIC and L5178Y/DIC lymphoma cells, might be more sensitive than the original cells to immunological damage. This hypothesis was recently indicated for a drug-resistant tumor system (16).

In order to test this hypothesis, the immunosensitivity of L1210 and L5178Y DIC-altered cells was evaluated. No increase in immunosensitivity was found. Neither cell-mediated nor humoral (complement-dependent) *in vivo* immunoresponse to DBA/2 histocompatibility antigens, as determined by *in vitro* assays, exerted differential cytotoxic activity against the original and the DIC-treated cells. Since the DIC treatment of L1210 and L5178Y did not modify the susceptibility to immunological damage, it is indicated that DIC treatment did not cause cell surface alterations leading to any nonspecific immunofragility.

With regard to the observation that allogeneic mice did not show a specific immune response to the DIC-induced

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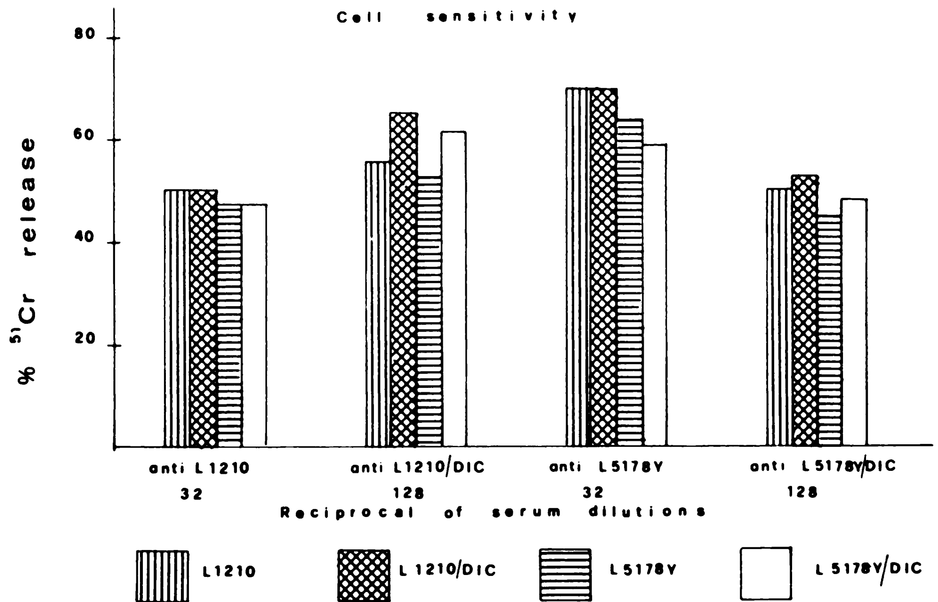

Chart 2. Target cell sensitivity to C3H antitumor sera.

Table 2
Inhibition of cell-mediated cytotoxicity by nonlabeled tumor cells

Effector spleen cells[a]			% reduction of cytotoxicity by adding (cells)[b]			
Donor strain	Immune to	Target cells	L1210	L1210/DIC	L5178Y	L5178Y/DIC
C3H	DBA/2	L1210	58	62	58	58
	DBA/2	L1210/DIC	73	77	81	80
	DBA/2	L5178Y	52	55	46	48
	DBA/2	L5178Y/DIC	58	53	54	53
	L1210	L1210	56	63	60	66
	L1210/DIC	L1210/DIC	51	58	61	64
	L5178Y	L5178Y	81	73	78	83
	L5178Y/DIC	L5178Y/DIC	71	73	71	60
C57BL/10	DBA/2	L1210	38	34	NT[c]	NT
	DBA/2	L1210/DIC	43	46	NT	NT

[a] 10^7 effector cells incubated with 5×10^4 ^{51}Cr-labeled target cells in presence of 2×10^5 unlabeled "inhibitor" cells.

[b] The reduction in lysis was calculated as follows:

$$\% \text{ reduction} = \frac{\% \text{ lysis without inhibitor cells} - \% \text{ lysis with inhibitor cells}}{\% \text{ lysis without inhibitor cells}} \times 100.$$

[c] NT, not tested.

antigen(s), it could be hypothesized that C3H and C57BL/10 mice are incapable of recognizing DIC-induced alloantigens of L1210/DIC and L5178Y/DIC leukemia sublines of DBA/2 origin [*e.g.*, lack of a specific immune response (Ir) gene]. Since cell-mediated immunity to DIC-induced antigen(s) was obtained in syngeneic animals (8) and a specific cytotoxic serum against cells of DIC-treated leukemia sublines was produced in rabbits following a heavy challenge of viable cells mixed with complete Freund adjuvant (Chart 4), the failure to detect an immune response in allogeneic mice could also be regarded as the result of the immunizing schedules and the evaluating techniques adopted in this study rather than as definite evidence of an absolute unreactivity of allogeneic mice to DIC-induced antigen(s).

New antigens associated with neoplastic transformation might be accompanied by a concomitant loss of the original histocompatibility antigens (4, 15). This possible occurrence has been tested for DIC-treated cells by inhibition assay of cellular cytotoxicity and by absorption of humoral cytotoxic activity. The data reported here provide evidence for the gross integrity of the histocompatibility antigens on the DIC cell surface. This point is in agreement with tumor transplantation studies (data not reported here): allogeneic animals, normal or immunodepressed, challenged with viable unaltered parental or DIC tumors did not show reduced capacity to reject DIC-treated tumors as compared with the parental tumors. The DIC tumors did not show any increased immunosensitivity (*in vitro*), and their cell kinetics

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Table 3
Decrease of cytotoxic activity of mouse antisera by absorption with original parental and DIC-treated tumor lines

Immune serum[a]			% reduction of cytotoxicity following absorption with (cells)[c]			
Donor strain	Immune to	Target cells[b]	L1210	L1210/DIC	L5178Y	L5178Y/DIC
C3H	DBA/2	L1210	40	39	29	20
	DBA/2	L1210/DIC	57	54	33	27
	DBA/2	L5178Y	44	43	58	57
	DBA/2	L5178Y/DIC	63	65	73	67
	L1210	L1210	86	85	78	85
	L1210/DIC	L1210/DIC	92	100	89	100
	L5178Y	L5178Y	87	85	91	82
	L5178Y/DIC	L5178Y/DIC	76	93	85	97
C57BL/10	DBA/2	L1210	59	57	57	52
	DBA/2	L1210/DIC	32	37	36	34
	DBA/2	L5178Y	56	49	64	56
	DBA/2	L5178Y/DIC	32	32	30	24

[a] C3H anti-DBA/2 serum was used at 1:32 dilution, all other sera were used at 1:16 dilution.
[b] 10^5 cells.
[c] Absorption was carried out at 37° for 30 min with occasional stirring. Cells (10^6) were used for absorption except for C3H antitumor antisera, which were absorbed with 5×10^6 cells. The % reduction in cytotoxicity was calculated as follows:

$$\% \text{ reduction} = \frac{\% \text{ lysis by nonabsorbed serum} - \% \text{ lysis by absorbed serum}}{\% \text{ lysis by unabsorbed serum}} \times 100.$$

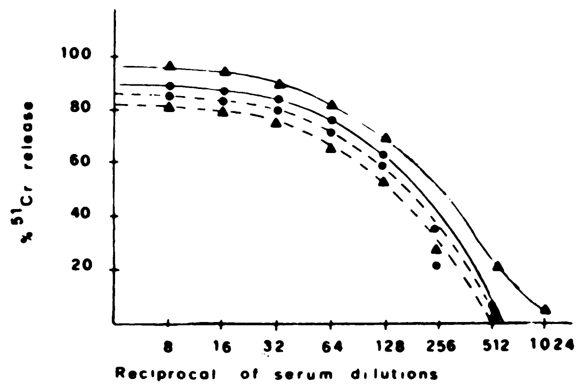

Chart 3. Target cell sensitivity to rabbit anti-L5178Y or anti-L5178Y/DIC sera. ●- - -●, L5178Y in anti-L5178Y serum; ●——●, L5178Y in anti-L5178Y/DIC serum; ▲- - -▲, L5178Y/DIC in anti-L5178Y serum; ▲——▲, L5178Y/DIC in anti-L5178Y/DIC serum.

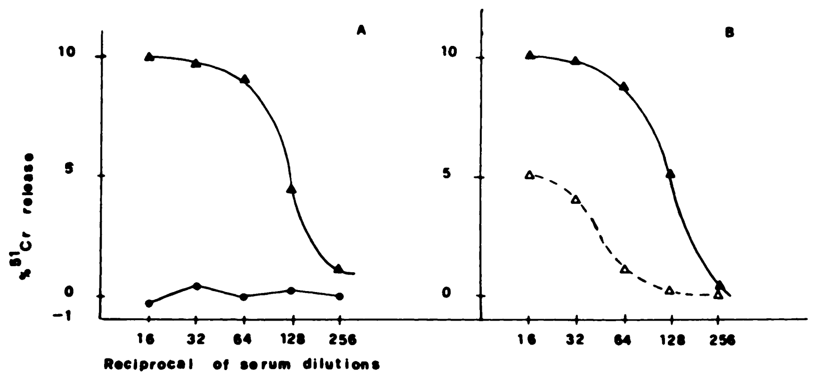

Chart 4. Activity of rabbit anti-L5178Y/DIC serum, absorbed with L5178Y, and its inhibition by L5178Y/DIC cells. *A*, 1:16 anti-L5178Y/DIC serum, absorbed with 100 × 10^6 L5178Y cells/ml was tested against L5178Y (●) or L5178Y/DIC cells (▲); *B*, the absorbed serum assayed against L5178Y/DIC cells. Two × 10^5 unlabeled (▲) L5178Y or (△) L5178Y/DIC cells were added to the test tubes.

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could not be distinguished from that of the original tumors (R. Silvestrini, unpublished data). Also, "DIC-related antigens" were not recognized by allogeneic recipients. Therefore, the rejection by the allogeneic host of DIC sublines would be explained on the basis of full expression of histocompatibility antigens.

In previous reports, evidence has been presented that DIC-treated tumor cells may possess altered antigenic characteristics (1, 7, 11). Although no generalization can be drawn, since the study was conducted with only 1 DIC subline (L5178Y/DIC), the serological data obtained in rabbits immunized with the parental or DIC subline lend additional support for the presence of DIC antigen(s) capable of eliciting a weak humoral-specific response in xenogeneic recipients.

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