The immune response to L 1210 leukemia is T-cell dependent

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ABSTRACT

Both untreated and neuraminidase-treated L 1210 cells stimulated proliferative activity of thymic lymphocytes in the BDF_1 host. This T-cell activity was followed by the production of immunoglobulins by B-cells. While neuraminidase-treated cells did not grow in the normal host, they did cause progressive tumor growth in the immunosuppressed hosts. In addition, the humoral response to either untreated or neuraminidase-treated tumor cells was inhibited when mice were immunosuppressed with rabbit anti-mouse thymocyte serum. Immunosuppressed mice were not able to respond to a T-dependent antigen, sheep red blood cells, but were able to respond to lipopolysaccharide, a T-independent antigen.

Neuraminidase-treated cells retained viability and redeposited sialic acid quickly after treatment with neuraminidase. The evidence indicates that competent T-cells were necessary for a humoral response to either untreated or VCN-treated tumor cells and that recognition of VCN-treated cells necessary for the production of a cytotoxic response occured within 4 to 5 hours after cells were injected.

INTRODUCTION

Virtually all animal neoplasms studied with sensitive techniques have been found to possess tumor-specific transplantation antigens (TSTA) which are absent from normal cells of the tumor-bearing host (1, 2, 3, 4). These antigens elicit a host response which can be measured by using a variety of assays (5, 6, 7). Both humoral and cell-mediated reactions against TSTA have been demonstrated with these techniques. However, tumors may

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continue to grow and metastasize in spite of the host response.

The host response to tumor cells may be modified by exposing tumor cells to a variety of treatments or agents prior to injection (8, 9, 10). Neuraminidase modified tumor cells elicit a different host response and these cells have been used to induce tumor immunity (9–11) and to cause complete remission of small, but progressively growing tumors (12, 13, 14). Differences in response to modified and unmodified cells may be dependent on differences in recognition and/or processing of tumor cell antigens. While recent evidence suggests that modified cells are recognized differently (Sansing and Kollmorgen, unpublished data), the role of thymus-dependent lymphocytes in mice given either untreated or VCN-treated cells is not clear. Consequently, a series of experiments were designed to elicit the importance of T-cells in the L 1210–BDF_1 tumor-host system.

MATERIAL AND METHODS

Tumor-host system

Male BDF_1 mice ($C57B1_6 \times DBA_2$) were 8 to 12 weeks of age and weighed about 20 grams (Sprague-Dawley, Madison, Wisconsin). L 1210 lymphoblastic leukemia cells were maintained as an ascites tumor by weekly serial transfer. Cultured L 1210 cells were grown in RPMI–1640, supplemented with 5% heat-inactivated, fetal calf serum, glutamine and antibiotics (Grand Island Biological Company, Grand Island, New York).

Treatment with neuraminidase

Cultured L 1210 cells were harvested during logarithmic growth, washed twice in 0.9% NaCl and incubated with *Vibrio cholerae* neuraminidase (VCN) (General Biochemicals, Chagrin Falls, Ohio) at 37°C for 1 hour. The final mixture was equal volumes of VCN (1 unit/10^6 cells) and acetate buffer at pH 5.6. The cells were then washed in 0.9% NaCl and viability was measured by trypan blue exclusion. VCN-cleavable sialic acid was determined by the thiobarbituric acid assay (15).

Immunosuppression

Mice were given an intraperitoneal injection of 0.1 ml rabbit anti-mouse thymocyte serum (RAMTS) (Microbiological Associates, Inc., Bethesda, Maryland) on 4 consecutive days, followed 3 days later by an additional injection of RAMTS and tumor cells. RAMTS was then administered at 3 day intervals for the duration of the study, and there was no evidence of RAMTS-induced toxicity.

The specificity of the RAMTS was evaluated by the Jerne plaque assay

(16) using sheep red blood cells (SRBC) and *Escherichia coli* lipopolysaccharide (LPS) as T-dependent and T-independent antigens respectively. Mice were given an intraperitoneal injection of either 4×10^8 SRBC (Colorado Serum, Denver, Colorado) or 40 μg of LPS from *E. coli* 0127 (Difco Laboratories, Detroit, Michigan). The number of plaque-forming cells (PFC) from spleens of immunized mice was determined 5 days later after exposure to SRBC or LPS-coated SRBC (17).

^{3}H-thymidine labeling of lymphoid cells

Mice were injected with untreated or VCN-treated tumor cells. Mice were killed at daily intervals (5 mice per group) for the duration of the study. Four hours prior to sacrifice, each mouse received an injection of ^{3}H-thymidine divided equally between tbe right and left inguinal regions (1 μCi/gram) (specific activity, 20 Ci/mmole, New England Nuclear, Boston, Mass.). A cell suspension was prepared from each thymus. Cells were washed 3 times in 0.01 M phosphate-buffered saline (PBS ; pH 7.2), transferred to glass slides and fixed in absolute methanol for 10 minutes, and layered with emulsion (NTB–2, Kodak, Rochester, New York). The slides were stored at 4°C for 3 weeks, developed, fixed and stained with Giemsa. The percentage of labelled cells was determined for a minumum of 1000 cells per slide.

Immunofluorescenc

Direct immunofluorescence was used to detect mouse IgG_1, IgG_2, and IgM on the surface membrane of spleen and peritoneal exudate cells. Single cells were obtained from whole spleens by aspiration in cold PBS (4°C). Cells were washed in cold PBS, smeared onto glass slides, air dried at room temperature, fixed in 95% ethanol for 5 minutes and rinsed with PBS. The slides were then covered with fluorescein-conjugated, goat anti-mouse immunoglobulin (1 : 5 dilution) (Meloy Laboratories, Springfield, Virginia) for 1 hour at 37°C in a moist atmosphere, then washed in cold PBS. Coverslips were attached with phosphate-buffered glycerol (pH 7.2) and cells were evaluated for fluorescence with an Olympus FLM–UV microscope, equipped with mercury vapor lamp (HB–200) and FITC interference filter. Cells were examined at 400× and a positive fluorescence was defined by speckled, cap, or ring fluorescence at the surface membrance (500 cells scored per slide).

Both cultured L 1210 cells and thymic lymphocytes lacked detectable membrance fluorescence using the above assay. In addition, cultured L 1210 cells incubated in either normal mouse serum or serum from mice bearing the L 5178 Y tumor cells also lacked membrane fluorescence, suggesting

that the immunoglobulins detected on tumor cells in the present study were tumor-specific.

The specificity of fluorescein-conjugated goat anti-mouse IgM, IgG_1 and IgG_2 was determined using purified mouse myeloma proteins (IgM, IgF, IgG, and IgH; Litton Bionetics, Inc., Kensington, Md.). Each of these proteins was tested against each of the goat anti-mouse immunoglobulins by Ouchterlony assays. After 48 hours, gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, Mo.). Each of the fluorescein-conjugated goat anti-mouse immunoglobulin sera reacted only with the corresponding mouse myeloma protein.

RESULTS

All mice injected with 10 or more L 1210 leukemic cells died of progressively growing tumor. The median survival time was inversely proportional to the logarithm of the initial tumor inoculum (Figure 1).

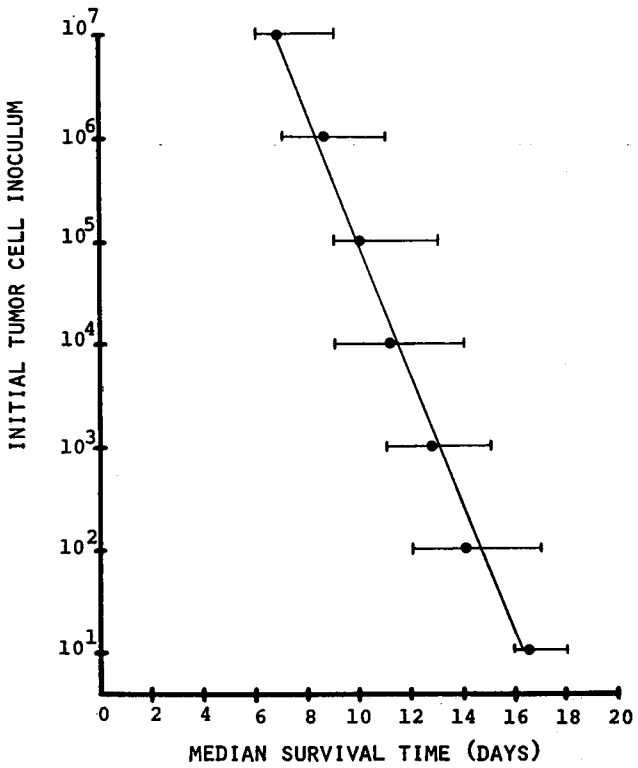

Fig. 1. Median survival time of BDF_1 mice as a function of initial inoculum of L 1210 cells. Horizontal bars indicate range of deaths. Each group consisted of 10 mice.

Tumor deaths did not occur and progressive tumor growth was not evident when mice were given 10^7 or fewer VCN-treated cells.

The viability of VCN-treated cells was evaluated by determing trypan blue exclusion, by measuring growth rate in culture, and by injection of these cells into immunosuppressed mice. Results are shown in Table 1. All of these parameters indicated that viability was retained after VCN treatment.

The host response to either untreated or VCN-treated tumor cells, as measured by the proliferative activity of thymic lymphocytes is shown in Figure 2. Proliferative activity was also measured by other parameters (Cantrell, Killion, and Kollmorgen, unpublished data) and confirmed with

Table 1. *Properties of neuraminidase-treated cells*

Treatment	Percent Excluding Trypan Blue	Doubling Time *in vitro*	Growth in normal mice	Growth in Immuno-suppressed mice
None	92%	16.1±0.9 hrs	yes	yes
Treated with VCN (pH=5.6)	87%	18.5±1.2 hrs	no	yes
Treated with heat inactivated VCN (pH=5.6)	89%	18.3±1.3 hrs	yes	yes

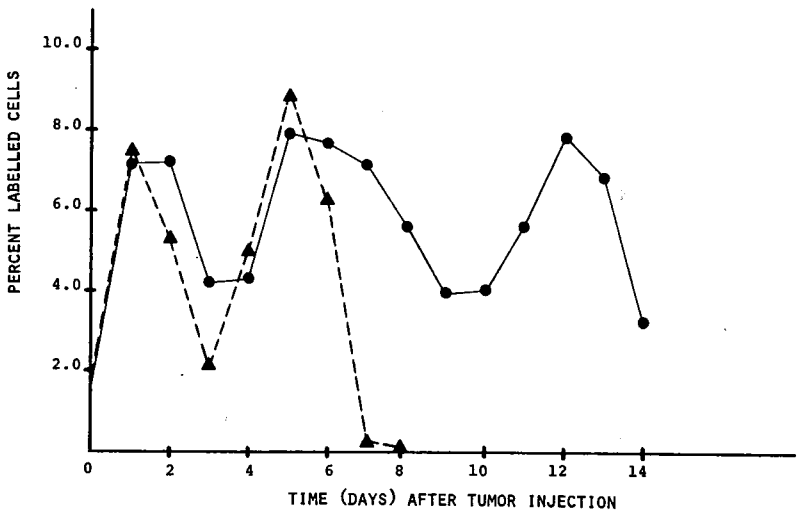

Fig. 2. Percent labelled cells in thymus as a function of time after injection of 10^6 untreated (▲- -▲) or VCN-treated (●—●) L 1210 cells. Mice were injected with ^{3}H-thymidine (1 μCi/gram body weight) 4 hours prior to sacrifice. All mice given untreated cells were dead by day 9. Points indicate mean values obtained using 3 to 5 mice.

histological evaluation (Glass, Glassell, and Kollmorgen, unpublished data). Based on percent labelled cells, the response to untreated and VCN-treated cells was similar for the first 5 days. Mice given untreated cells showed a progressive loss of labelled and total thymic lymphocotes and all mice were dead by day 9. Histological evaluation indicated thymic atrophy prior to death. While tumor cells metastasized to various tissues and organs, there was no evidence of tumor cells in the thymus. Mice given VCN-treated cells had a third peak of proliferative activity on day 12.

Mice given either untreated or VCN-treated cells produced immunoglobulins which were observed on both spleen and peritoneal exudate cells. Figure 3 shows the percent of spleen and peritoneal exudate cells with various species of surface bound immunoglobulins as measured by immunofluorescence. The host response to untreated cells, which produced progressive tumor growth, was primarily IgG_1 and IgG_2. On the other hand, the response to VCN-treated cells was primarily IgG_2 and IgM.

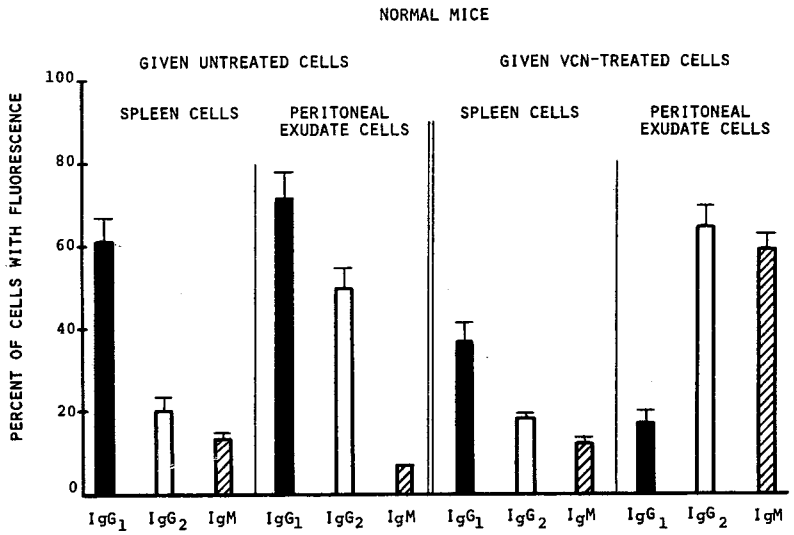

Fig. 3. Percent of spleen and peritoneal exudate cells bearing membrane-associated immunoglobulins 8 days after injection of 10^6 untreated or VCN-treated L 1210 cells into normal BDF_1 mice. Cells removed from 6 to 12 mice were examined. Three slides (500 cells/slide) were evaluated per mouse. Standard error is shown when visually distinguishable from the mean.

Figure 4 illustrates that the response to either untreated or VCN-treated cells was markedly inhibited in mice treated with rabbit anti-mouse thymocyte serum. While immunosuppressed mice did not respond to sheep

red blood cells, they were able to respond to lipopolysaccharide (Table 2).

Another series of experiments indicated that sialic acid was redeposited within hours after cleavage with neuraminidase. The data in Figure 5 shows the rate of redeposition on cultured cells. Figure 6 illustrates that

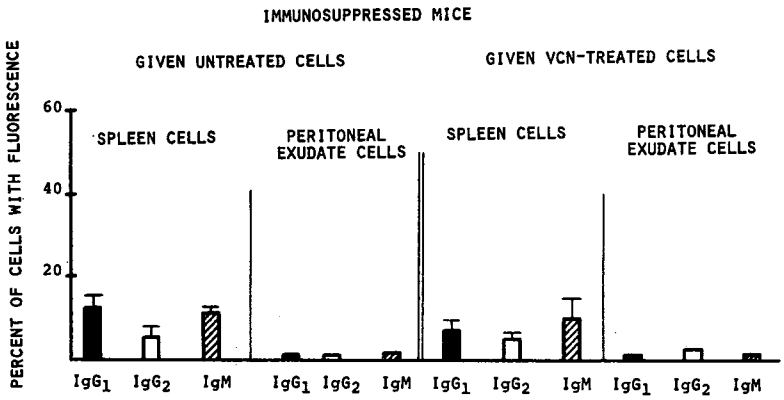

Fig. 4. Percent of spleen and peritoneal exudate cells bearing membrane-associated immunoglobulins 8 days after injection of 10^6 untreated or VCN-treated L 1210 cells into immunosuppressed BDF_1 mice. Mice were immunosuppressed with rabbit anti-mouse thymocyte serum. Cells removed from 3 to 6 mice were examined. Three slides (500 cells/slide were evaluated per mouse. Standard error is shown when visually distinguishable from the mean.

Table 2. *Immunosuppressive effect of whole-body irradiation or RAMTS on the immune response to SRBC and lipopolysaccharide*

Immunosuppressive treatment	Immunogen	Number of mice	Number of PFC/10^6 spleen cells to SRBC (±S.E.)
None	SRBC	4	249.5±23.5
None	None	2	5.0± 0.7†
RAMTS	SRBC	4	18.5± 4.5†
Immunosuppressive treatment	**Immunogen**	**Number of mice**	**Number of PFC/10^6 spleen cells to LPS (±S.E.)**
None	LPS	4	123.3± 5.6
None	None	2	3.5± 2.0
RAMTS	LPS	4	114.3± 8.4*

Student-t test was used to determine significant differences.

† significant ($P<0.001$) compared to normal mice immunized with SRBC.

* not significant ($P>0.05$) compared to normal mice immunized with lipopolysaccharide.

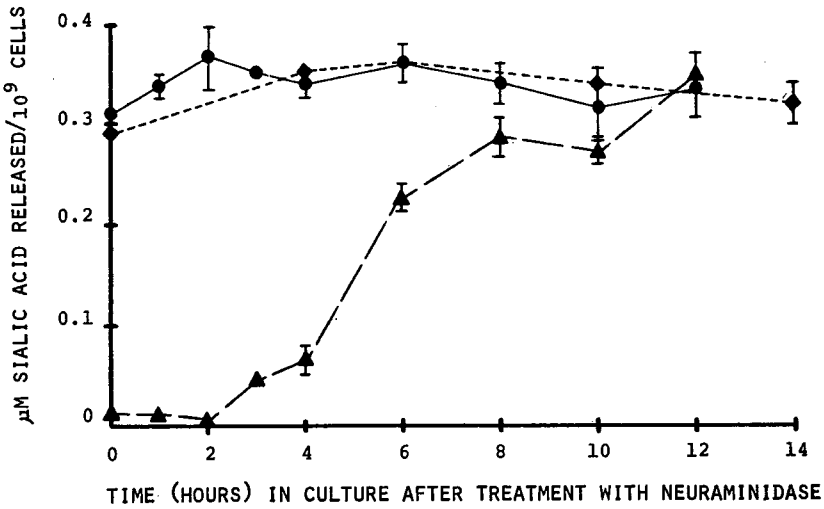

Fig. 5. Regeneration of VCN-cleavable sialic acid on L 1210 cells as a function of time after VCN-treatment (▲— —▲) or sham-treatment. Sham-treatment consisted of treatment with acetate buffer (●——●) or heat-inactivated VCN (◆- - -◆). Points indicate average amount of sialic acid released by subsequent VCN-treatment. Quadruplicate samples were evaluated per point. Standard errors larger than symbol dimensions are shown.

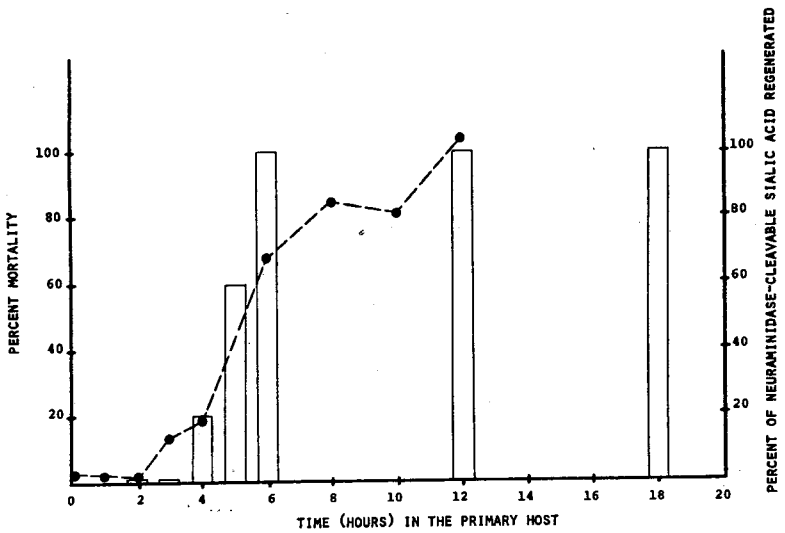

Fig. 6. Percent of VCN-cleavable sialic acid regenerated on L 1210 cells as a function of time in culture after initial VCN-treatment (●- - -●). Percent mortality of secondary hosts (bars) as a function of residence time of VCN-treated L 1210 cells in the primary hosts. Five secondary hosts were evaluated per time point.

VCN-treated cells removed from primary hosts did not cause progressive tumor growth and death of all secondary hosts until after a six hour residence time in the primary host. These data correlated well with redeposition of sialic acid on cultured cells and emphasized the transient nature of neuraminidase induced changes.

DISCUSSION

Immunosuppression produced by treatment with rabbit antiserum to mouse thymocytes is essentially equivalent to the immunosuppressive effects caused by neonatal thymectomy (18). Both techniques suppress antibody production to T-dependent antigens. However, it is not clear if a reduction in lymphocytes or the absence of a humoral thymic factor controls immunocompetence (19).

The specificity of rabbit antiserum to mouse thymocytes was confirmed by observing the response to a T-dependent antigen, sheep red blood cells, and to a T-independent antigen, lipopolysaccharide (20). These observations indicated that competent B-cells were present in both normal and immunosuppressed mice.

Normal mice were able to produce antibodies to either untreated or neuraminidase-treated tumor cells. Unlike normal mice, immunosuppressed mice were not able to reject neuraminidase-treated tumor cells, and did not produce antibodies to either cell type. The difference in response to untreated and treated cells could not be attributed to an alteration in T-cell dependency. Similarly, athymic nude mice do not respond to T-dependent antigens unless thymic function has been restored (21). Other studies have demonstrated that neuraminidase-treated cells grow as untreated cells in athymic mice (Kollmorgen, unpublished data).

Neuraminidase induced transient changes in the properties of the cell surface membranes. Treated cells redeposited cell surface sialic acid quickly (22, 23) and behaved as normal cells within several hours after treatment. Therefore, any differences in the host response to treated and untreated cells must necessarily be initiated during the brief interval when the host could distinguish between untreated and treated cells.

Differences in recognition very likely lead to differences in response. It is well established that neuraminidase modified cells can be used to induce tumor immunity (11) and to cause complete remission of small, but progressively growing tumors with similar antigenic properties (12). Antigenic topography is probably of considerable importance in determining the host response. Removal of sialic acid may only constitute a necessary requisite for subsequent topographical rearrangements. We have shown

that some cell clones are highly antigenic in spite of their cell surface sialic acid. Treatment of these highly antigenic cells with neuraminidase abrogates their antigenic properties (Killion and Kollmorgen, unpublished data).

Our findings are consistent with the notion that the host response was dependent on initial recognition and that tumor antigens may be recognized differently depending on their molecular environment. While this environment is critical for recognition, it is not critical for antibody absorption. Finally, modification of tumor cells, which leads to differences in recognition does not alter the necessity of competent T-cells for the subsequent production of tumor specific antibodies.

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