Effect of Progressive Neoplastic Growth on the Decarboxylation of DL-[1-14C]Ornithine by Lymphocytes from C3H/He Tumor Hosts

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ABSTRACT

The reactivity of normal and tumor host lymphocytes incubated with normal serum or with serum or malignant ascites fluid from tumor hosts was measured by the ability of the lymphocytes to synthesize ornithine decarboxylase after phytohemagglutinin stimulation. Each of three tumors tested (a solid fibrosarcoma, an ascites mammary carcinoma, and an ascites ovarian carcinoma) caused increasing unresponsiveness in the lymphocytes of mice with progressing syngeneic neoplastic growth. The sera and particularly the malignant ascites fluids from mice given implants of the ascites cancers became progressively inhibitory to the activation of lymphocytes from tumor hosts as well as from normal mice. The serum from mice carrying s.c. implants of the fibrosarcoma enhanced the activation of lymphocytes from tumor hosts and from normal mice during early tumor growth but it also became inhibitory.

INTRODUCTION

Previous publications have described how specific transplantation resistance developed with the growth of a sensitizing tumor implant and then declined as the growing tumor exceeded a certain critical size (26, 30). The declining resistance was related to the accumulation of soluble tumor factors, presumed to include antigens, in the blood (28). The specific resistance was quickly recovered once the source of excess antigen was removed (26, 29). It appeared that the liver was particularly important in clearing immunosuppressive factors from the blood (25).

Several investigators have found that the serum of cancer patients or animal tumor hosts may suppress the in vitro responsiveness of lymphocytes. Specific and nonspecific factors have been implicated. An extensive study of nonspecific suppressive factors in cancer patients was reported by Glasgow et al. (10) in which one-half of the patients had significant concentrations of a serum factor that reduced the activation of normal lymphocytes by PHA. Hellström and Hellström (11) reported that serum from mice bearing a methylcholanthrene-induced sarcoma contained T-cells capable of suppressing a tumor rejection response in adoptive transfer recipients.

In the experiments described here, the responsiveness of lymphoid cells from normal mice and from tumor hosts was determined by the ability of the cells to increase ODC synthesis when stimulated with PHA. The effect of tumor host serum or malignant ascites fluid on lymphocyte responsiveness was then studied at progressive stages of neoplastic growth.

Increased levels of polyamines and their biosynthetic enzymes (including ODC) are associated with growth and mitotic activity (2, 7, 23). The increase usually precedes increases in RNA, DNA, and protein levels. ODC activity has been shown to parallel RNA synthesis in a variety of rapidly dividing cells (6, 19, 20) and in lymphocytes activated with PHA (13). Because increased ODC activity is associated with increased synthetic activity in cells preparing to undergo mitosis, monitoring ODC activity provides a method to measure viability and replication in populations of cells. This report describes the use of this approach to measure the in vitro ODC activity of intact lymphocytes, which is used as a gauge of the immune competence of the tumor host.

MATERIALS AND METHODS

Animals. The experimental animals were female C3H/He and C3Hf/He mice, 8 to 12 weeks old at the start of the experiments. The mice were raised and kept in a pathogen-free barrier colony.

Tumors. The FS had been induced s.c. in a female C3H mouse by methylcholanthrene and kept in liquid nitrogen. It was reintroduced into syngeneic mice to be used in these experiments in the 6th to 11th transplant generations. The MCA had developed spontaneously in a multiparous C3H mouse and had been converted to the ascites form after 2 i.p. passages in syngeneic C3H mice. The OVCA had developed spontaneously in a multiparous C3H mouse and had been converted to the ascites form after 6 i.p. passages in syngeneic C3Hf mice. The conver-
sions had been facilitated by the addition of 0.25 ml of cell-
free ascites fluid per 10^6 cells implanted i.p.

Tumor Implantation. The malignant ascites cells were
sedimented in the centrifuge, and the pellet was washed 2
times in “assay medium” made of Dulbecco’s phosphate-
buffered saline with 0.03% bovine serum albumin (Grand
Island Biological Co., Grand Island, N. Y.) and P/S.

FS tissue was removed from freshly killed or live anesthe-
tized donor animals, and two 1-cm pieces of living tumor tissue were implanted s.c. in the right flank to initiate
new growth.

Lymphocyte Preparations. For determination of the in-
fluence of progressive tumor growth on the reactivity of
mouse lymphocytes in vitro, spleen cells (90 to 95% mono-
nuclear cells) from tumor hosts and lymph node cells (99%
mononuclear cells) and spleen cells from normal mice were
used in the tests.

Spleen cells were prepared in single-cell suspensions by
forcing minced spleen tissue through 105 mesh polyester
cloth (HC 7-105; Tetco, Elmsford, N. Y.) into ice-cold RPMI
1640 (Associated Biomedics, Buffalo, N. Y.) containing 10%
fetal calf serum (Grand Island Biological Co.) plus P/S. The
mechanical separation of single cells from solid tissue has
been described in detail in previous publications (26, 27).
The suspension of single cells separated from the spleens
was then centrifuged and washed (150 x g for 10 min) 3
times in assay medium. The pellet was resuspended in
assay medium to a concentration of 10^6 cells/ml.

Lymph node cells were prepared in single-cell suspensions
by mincing the inguinal, brachial, and axillary nodes in ice-
cold RPMI 1640. Single cells were freed from the minced
by gently and repeatedly drawing the pieces into a 1-
ml syringe. The single cells were washed 3 times in assay
medium and resuspended to a concentration of 10^6 cells/
ml.

[^3H]dThd Uptake Assay. The lymphocytes were incu-
bated in sterile disposable 16 x 100-mm Pyrex culture
tubes containing 2 ml of RPMI 1640 plus 10% fetal calf
serum and P/S at 37°C, 95% humidity, and 5% CO2. The
incubation process was terminated with the injection
of 0.2 ml of RPMI 1640 containing 0.05 M pyridoxal phospha-
tic activity, 29 mCi/mmol; New England Nuclear),
0.05 M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.). PHA (Wellcome Inc., Research
Triangle Park, N. C.), 2 ml/ml, was added at this time.
The ornithine and 50 /¿I of heat-inactivated serum or
ascites fluid were added at the beginning of the assay.
Dose-response curves with points at 5, 10, 25, 50, 100, 200,
and 500 /¿I were established for at least 1 sample of each of
the peritoneal fluids and sera tested. Each sample showed
increasing activity from 5 to 25 /¿I, and each demonstrated
declining activity at or above 200 /¿I. Accordingly, the
quantity used in each of the experiments reported here was
50 /¿I. The tubes were capped with a sleeve-type rubber cap
(A. H. Thomas Co., Philadelphia, Pa.) perforated to hold the
stem of a plastic center well (Kontes Glass Co., Vineland,
N. Y.). The tubes were incubated at 37°C, 5% CO2 humidified,
for 20 hr (except in studies to determine the optimum time
of incubation).

The incubation process was terminated with the injection
of 0.2 ml of RPMI 1640 plus 10% fetal calf serum and P/S at
37°C, 95% humidity, and 5% CO2.

The spontaneous release of 14CO2 was determined in
control samples containing acetic-acid-fixed lymphocytes,
and the cpm were subtracted from the activity measured in
experimental groups. All samples were tested in triplicate.

The measurable ODC activity in unstimulated and PHA-
stimulated lymphocytes up to, on the average, 7 x 10^6 cells.
Greater numbers of cells produced insignificantly greater
release of 14CO2. Accordingly, the standard quantity elected
was 10^7 cells in all of the tests reported here.

Spleen or lymph node cells (10^7) were incubated in sterile
disposable 16 x 100-mm Pyrex culture tubes containing 2
ml of RPMI 1640 plus 0.1 /¿Ci of [3H]dThd (specific activity, 6.7 mCi/mmol; New England Nuclear),
0.05 M pyridoxal phosphate (Sigma Chemical Co., St.
Louis, Mo.), and 0.05 M 2-mercaptoethanol (Eastman
Kodak Co., Rochester, N. Y.). PHA (Wellcome Inc., Research
Triangle Park, N. C.), 2 ml/ml, was added at this time.
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of incubation).

The incubation process was terminated with the injection
of 0.2 ml of RPMI 1640 plus 10% fetal calf serum and P/S at
37°C, 95% humidity, and 5% CO2.

The center wells were removed with the rubber cap and
carefully snipped off into scintillation vials containing 10 ml
of scintillation fluid made from Liquifluor (New England Nuclear) and Scintanalysed toluene (Fisher Scientific Co.,
Fair Lawn, N. J.). The vials were gently shaken for 30 min
before the activity was counted in a Beckman LS-230 liquid
scintillation counter.

The spontaneous release of 14CO2 was determined in
control samples containing acetic-acid-fixed lymphocytes,
and the cpm were subtracted from the activity measured in
experimental groups. All samples were tested in triplicate.

The ODC activity in unstimulated and PHA-
stimulated spleen and lymph node cells was always signifi-
cantly reduced when the extracts [by the method of Russell
and Snyder (21)] of sonically disrupted cells rather than
intact cells were assayed.

Malignant cells, ascites fluids, and sera were removed at
progressive stages of malignant growth. Cells and fluids
were removed and tested together on the same day. For
prolongation of the life of mice given implants i.p. of
malignant ascites cells, paracentesis was performed at 3
and at 4 weeks after implantation. Accordingly, all cells and
fluids removed from these tumor hosts after Day 21 were
taken after paracentesis.

All animals were killed by cervical dislocation.
RESULTS

Lymphocytes were treated with the following heat-inactivated fluids at the time that they were stimulated with PHA: normal mouse serum; serum from mice carrying s.c. FS implants; serum and malignant ascites fluid from mice carrying ascites MCA implants; and serum and malignant ascites fluid from mice carrying ascites OVCA implants.

Tests with Lymphocytes from Normal Mice. The points on the curve presented in Chart 1 each represent the average of 20 tests. The results show that the unstimulated ODC activity (no PHA added) of spleen cells and lymph node cells was depressed in the presence of tumor host serum. By the fourth week after tumor implantation, the host serum reduced the ODC activity to the lowest level of sensitivity of the system (spontaneous release of $^{14}$CO$_2$ in control samples with acetone-fixed lymphocytes). (The 3 curves for unstimulated cells treated with the 3 tumor host sera were so close that they were combined into a single line in Chart 1.)

With the addition of PHA to the cell suspensions, it became apparent that the progressive growth of the 3 different cancers resulted in the appearance in the host serum of factors that had similar but for each tumor quantitatively distinguishable effects on lymphocyte activation. The s.c. growing FS produced at first a stimulating effect and then a progressively suppressive effect. The ascites MCA produced a mere suggestion of an initially stimulating effect before becoming suppressive. The ascites OVCA produced, from the first test 2 days after implantation, only suppression of lymphocyte activation.

The question of whether in FS hosts the production of an early stimulating factor was suppressed by progressive tumor growth or was overcome or neutralized by an inhibitor is a complex problem currently being investigated.

By comparing the viability (trypan blue exclusion) of lymphocytes incubated with normal serum with the viability of lymphocytes incubated with host serum or ascites fluid collected at progressive stages of malignant growth, it appeared that the suppression of ODC synthesis was not a result of cell destruction. In no cell sample stained with trypan blue was the proportion of completely unstained cells less than 75% at the end of the 20-hr incubation period.

Because interaction between serum factors and PHA could possibly interfere with PHA binding and activation of lymphocytes, normal spleen cells were preincubated with PHA for periods ranging from 30 min to 8 hr and then were incubated with serum obtained from hosts 15 days after MCA ascites implantation. Cell suspensions preincubated with PHA did not respond differently from cell suspensions treated simultaneously with PHA and serum.

Tests with Lymphocytes from Tumor Hosts. Chart 2 shows the average results of 12 tests each with FS and MCA and 10 tests with OVCA. The basic ODC activity (no PHA added) of washed, untreated spleen cells from the hosts of all 3 cancers declined with progressive growth, but the spleen cells from FS hosts showed an initial phase of increased activity.

Responsiveness of tumor host spleen cells to PHA activation declined gradually with progressing disease. The addition of normal serum produced the well-recognized effect of modulation of lymphocyte activation. The addition of normal serum also revealed that the spleen cells from the
hosts of all 3 tumors, even the rapidly suppressive ascites OVC, had an initial (Day 4 after tumor implant) although only very slightly increased reactivity to PHA.

The host sera and, particularly, the ascites fluids from mice carrying the MCA and the OVCA became rapidly and strongly suppressive with progressing disease. Serum from mice carrying the FS, which up to the 16th day of s.c. growth had increased the PHA response of washed spleen cells, thereafter added to the loss of reactivity.

The decline in reactivity was related to malignant growth and not to age, since in a few parallel control tests with spleen cells from normal mice of the same age and sex as the tumor hosts no decline in reactivity was detected.

Separate tests (data not presented) determined that the optimum time of ODC measurements, whether the lymphocytes were cultured without serum or in normal serum, host serum, or ascites fluids, in most cases occurred before 24 hr of incubation. Accordingly and for convenience, a 20-hr period of incubation was selected for all of the tests reported here, except the tests comparing ODC activity and [3H]dThd uptake (Chart 3).

Comparison of ODC Production and Thymidine Uptake. To compare and relate the tests for ODC production in stimulated lymphocytes to the thymidine uptake assay for lymphocyte replication, we did some of the tests as parallel tests of both ODC production and [3H]dThd uptake using cells from the same suspensions. Chart 3 represents the average values of 3 tests that compared, in parallel, the development of ODC activity with the thymidine uptake in PHA-stimulated C3Hf spleen cells for up to 168 hr after the addition of 5 µl of PHA per ml. The curves show that net ODC activity was maximum at 24 hr and had returned to normal at 72 hr. Thymidine incorporation was significantly above normal 96 hr after stimulation and continued to increase for the duration of the test. The viability (trypan blue exclusion) of both normal and stimulated cells remained above 90% for the 168 hr of observations.

The graphs show that the ratio of stimulated to unstimulated ODC activity in spleen cells at 24 hr was 2300:400 cpm, or 5.8. The same evaluation of [3H]dThd uptake at 96 hr was 2000:400, or 5; at 120 hr the ratio for [3H]dThd uptake was 4250:500, or 8.5. In other words the sensitivity of the 24-hr ODC assay for spleen cells activated by PHA was between the sensitivity of fourth-day and fifth-day [3H]dThd uptake assays.

DISCUSSION

The results of this investigation have shown that the level of production of the enzyme ODC can be measured in living lymphocytes and used to indicate changes in lymphocyte responsiveness to mitogens. Since ODC specifically converts ornithine to putrescine by decarboxylation at position 1 (21), it is less probable that the 14CO2 was produced after the [1-14C]ornithine had been converted by deamination to glutamate and had entered the Krebs cycle.

The factor(s) that appeared in the serum of tumor hosts and in their malignant effusion fluids had, with progressive malignant growth, a profoundly suppressive effect on normal lymphocyte responsiveness (Chart 1). In the lymphocytes of tumor hosts, both the basic ODC activity in unstimulated cells and the response to PHA stimulation measured by increased ODC production were reduced as the malignant growth progressed. This declining ODC activity could be further reduced by incubating tumor host lymphocytes in host serum or ascites fluid (Chart 2). The results presented in Chart 3 show that increased ODC production preceded by about 72 hr and paralleled [3H]dThd uptake during lymphoblast transformation. This is in agreement with studies in other cell systems (2).

Other serum factor(s) also seemed to be involved, which appeared early in malignant growth and increased, although in some cases very briefly, the responsiveness of lymphocytes to PHA stimulation (Charts 1 and 2). This shift from "positive" to "negative" factors during tumor progres-
Lymphocyte ODC with Tumor Progression

Chart 3. Net ODC activity and thymidine uptake in unstimulated and in PHA-stimulated normal mouse spleen cells after increasing periods of incubation. Each cpm value is the mean of 3 replicates, corrected for spontaneous 14CO2 release and for spontaneous [3H]dThd adsorption. Numbers in parentheses, ratios of the cpm values of stimulated cells to the cpm values of unstimulated cells.

The nature of the negative factor(s) that reduced the ODC response equally in lymphocytes taken from tumor hosts or from normal mice is not yet known. Evidence has been presented that the serum factor that affects the functional capacity of the lymphocytes of animal tumor hosts or cancer patients may be (a) antibody or antigen-antibody complex (12, 22), (b) free antigen (3), (c) α-globulin (10, 18), or (d) a peptide fraction of α-globulin (10).

The stronger and earlier depressive effect of ascites fluid compared to host serum (Chart 2) suggests that some of the factors were derived from malignant cells. The removal by paracentesis of ascites fluid and malignant cells did not suffice, however, to relieve the progressive depression of lymphocyte responsiveness.

In addition to substantial evidence from clinical and experimental studies that general anergy is associated with advanced cancer, there may also, with antigenic neoplasms, be a more rapidly effective reduction of specific resistance factors through neutralization by free tumor antigen (24, 28). Accordingly, the reduced responsiveness seen in normal lymphocytes incubated with tumor host serum may have been caused by a variety of soluble toxic as well as functional factors, including soluble immunoregulators. In the experiments with tumor host lymphocytes, an additional effect by suppressor cells present among the lymphocytes (5, 9, 14-16) must also be considered.

The nature of the positive serum factor(s), which early in malignant growth increased lymphocyte activation, is also not known. A variety of physiological conditions and factors can produce changes in the hydration of glycoproteins and proteoglycans on the cell surface that will influence the selective passage of cations, particularly Ca++, into the cells. The entry of cations into the cytoplasm and the nucleus is closely related to the events that activate cells and move them toward replication. The biochemical changes that occur in stimulated lymphocytes have been recently reviewed by Wedner and Parker (31).

The conditions and events presented in this paper may suggest a point of similarity in the relationships of hosts to neoplastic and infectious diseases. With progressive neoplastic disease a condition may develop in which, analogous to lobar pneumonia before sulfonamides, the parasite-malignant condition avoids resolution by shedding material that can neutralize host resistance by precluding effector cell activation, and/or by neutralizing active resistance factors.

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REFERENCES

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