Immunological Studies with the Use of Nutritional Variants of Sarcoma 180

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SUMMARY

Two cell lines of Sarcoma 180 (S-180), CCRF-I and CCRF-II, which do not require added tryptophan for growth in cell culture were studied for their growth characteristics in vivo. S-180 (CCRF-I) regressed rapidly when implanted subcutaneously in pathogen-free mice. The mice whose tumors had regressed were resistant to subsequent challenge with the more virulent S-180 (TUC) and Ehrlich's carcinoma tumors.

Preliminary serologic studies using complement fixation and anaphylaxis tests showed that rabbit antisera to CCRF-I and TUC-S180 cells reacted differently when tested against whole cell antigens. The differences noted in these studies may be quantitative in nature and due to changes in cell surface protein.

Attempts to produce immunity in mice by pretreatment with tumor cells irradiated with x-rays in vitro began with studies by Contamin (4) in 1910. Goldfeder (10), McKee (18), and Donaldson (5) studied the effect of x-irradiation on tumor cells and produced varying degrees of immunity to S-180 and Ehrlich's carcinoma by pretreatment with x-irradiated tumor cells. Donaldson and North (6) showed that multiple injection of nitrogen mustard-treated Ehrlich's carcinoma cells resulted in greater immunity against subsequent tumor challenge. MacDowell (14) was able to immunize mice to a transplantable leukemia by a "desensitization" technic using small numbers of viable cells.

The development of cloning technics has led to the isolation of cell lines which could then be studied for their nutritional requirements, metabolic pathways, and other characteristics. Nutritional variants of serially propagated human cell lines have been described by numerous workers (2, 3, 20, 22). Two cell lines of Jensen sarcoma, a rat tumor, were isolated by McCoy and co-workers (17) and were shown to differ in their growth requirement for asparagine.

Mutants of a given cell line, which may have arisen from either x-irradiation or changes in their nutritional requirements, may show such diverse metabolic changes that these may be reflected not only in their growth rate in vivo, but also in their immunologic and serologic relationships. A culture of S-180, which does not require added tryptophan for growth in cell culture, and two tryptophan-requiring strains were studied with respect to their in vivo growth-potential and serologic relationships. One of the cell lines, CCRF-I, which does not require added tryptophan for growth, regressed rapidly when implanted subcutaneously in specific pathogen-free mice. The mice whose tumors had regressed were resistant to subsequent challenge with the more virulent Upjohn strain (TUC) of S-180 and with Ehrlich's carcinoma. Since the cell lines used in this study have been transferred for many generations, the resistance observed may reflect differences in the antigenicity of the cell lines rather than the presence of tumor-specific antigens. Preliminary studies using antisera prepared against the two S-180 lines are reported in this paper.

MATERIALS AND METHODS

CCRF-I and CCRF-II, two strains of S-180 which do not require added tryptophan for growth in cell culture, were received from Dr. George Foley and have been characterized previously (8). The two CCRF strains were grown in Eagle's tissue culture medium, supplemented by 10% horse serum, using either stationary flasks or large roller bottles. The cells were scraped from the glass at the end of the growth phase, centrifuged and resuspended in physiologic saline for experimental studies.

The Upjohn strain of S-180 (TUC-S180) was received from Dr. K. Sugiura and was carried in its ascitic form in Swiss mice by transfer at weekly intervals. The Upjohn strain of S-180, was shown by Foley to require added tryptophan for growth in vitro (8). The Ehrlich's carcinoma was obtained from Dr. T. Hauschka of Roswell Park Memorial Institute and was carried as an ascitic tumor in Swiss mice. The tumor cells were suspended in saline and counted using standard hemacytometer technic. Viability of the cells was checked using Schrek's technic (21). Female Swiss mice (18—22 gm) from the Upjohn pathogen-free stock colony were used in this study.

RESULTS

In vivo growth studies.—CCRF-I and TUC-S180 cells were tested for their ability to grow as a solid tumor.
Body weights and tumor measurements (average of 2
Each line of 5-180 was implanted subcutaneously in the
groin of twenty mice using an inoculum of 340,000 cells.

Nineteen of the mice which had received the CCRF-I
cells were alive 31 days later. The tumors of twelve of the
mice had regressed completely. Tumors were regressing
in four mice and did not take in three others. In contrast,
all of the mice receiving the TUC-S180 cells developed
tumors which grew progressively with no evidence of re-
gression (Chart 1).

The nineteen mice which had received the CCRF-I cells
and had survived for 31 days were challenged subcu-
taneously with 500,000 TUC-S180 cells. Six of the
twelve mice whose original tumors had regressed com-
pletely did not accept the challenge tumor. Two de-
veloped small tumors which regressed rapidly and the re-
maining four developed tumors which grew at the same rate
as those of the controls. The three mice which did not
accept the original tumor did not accept the challenge.
The four mice whose original tumors (CCRF-I) were re-
gressing at the time the challenge dose of TUC-S180 was
given, permitted the challenge tumor to grow even though
the original tumor continued to regress. The results of the
experiment indicated that CCRF-I cells will grow as a
solid tumor in Swiss mice, but that the mass will regress
completely in a high percentage of the animals. Regres-
sion of the CCRF-I tumor led to the development of
demonstrable immunity to the more malignant TUC-S180
tumor.

The two lines of S-180 were compared for their ability to
grow in ascitic form. Each cell line (CCRF-I and TUC-
S180) was injected intraperitoneally into groups of twenty
mice each, using a tumor inoculum of 340,000 cells. The
mean survival time was 13.5 days for the mice which had
been implanted with the TUC-S180 cells. Only one of the
twenty mice which had received the CCRF-I cells de-
veloped an ascitic condition and no solid tumors were de-
tected during the 31-day observation period. In contrast,
all of the mice which received TUC-S180 cells I.P. de-
veloped tumors. The nineteen surviving mice were chal-
lenged with 50,000 TUC-S180 cells subcutaneously and
observed for an additional 24 days. No significant dif-
fERENCE appeared in either the number of ‘no takes’ or tumor
size, between the ‘pretreated’ group and a control group of
untreated mice of the same age and sex. A single exposure
of mice to CCRF-I cells I.P. did not induce the same
degree of immunity to TUC-S180 cells as the S.C. in-
jection of the same cells.

**Immunization studies with CCRF-I cells.**—The results of
the first experiment, in which mice that had received a
single S.C. implant of CCRF-I cells showed resistance to a
subsequent challenge with TUC-S180 cells, suggested that
a greater degree of immunity might result from repeated
antigenic exposure to the CCRF-I cells. When tested ex-
perimentally, it was found that pretreatment with re-
peated antigenic exposure to CCRF-I cells led to the de-
velopment of immunity not only to TUC-S180 but also to
Ehrlich’s carcinoma cells (Chart 2).

Eighty mice were treated subcutaneously 6 times with
CCRF-I, starting with 50,000 and increasing to 200,000,
cells, each at a different site. All mice were weighed at
the start of the experiment; at 7-day intervals thereafter
they were weighed and each treatment site checked for
tumor growth. About 50 % of the mice showed edema and
swelling at the site of the first two injections of cells. The
swelling disappeared and only one measurable tumor de-
veloped from the first six cell treatments in 80 mice. The
80 mice were divided into two subgroups of 40 each. One
subgroup was challenged subcutaneously with 4.5 million
CCRF-I cells and the other subgroup challenged with the
same number of TUC-S180 cells. Control groups, each
containing twenty untreated mice, received the same
treatment of mice of the same age and sex. A single exposure
to CCRF-I cells I.P. did not induce the same
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same number of TUC-S180 cells. Control groups, each
containing twenty untreated mice, received the same
number of challenge cells. The failure of the CCRF-I cells
to grow in the control group may be attributed to the
older age of the control mice as well as the poor in vivo
growth characteristics of the cell line, as shown in Chart 1.
The subgroup which had been challenged with CCRF-I
cells and observed for 49 days, with no tumors developing,
was then challenged with 4.0 million TUC-S180 cells and
observed for an additional 38 days. A significant difference
(15) between the control group of twenty mice and the
pretreated group with respect to the number of ‘no takes’
(P = 0.01) was shown.

The other subgroup of 40 pretreated mice and a group of
twenty untreated controls of the same age and sex were
challenged with 4.5 million TUC-S180 cells and observed
for 61 days. A significant difference (P = 0.01) between
the number of ‘no takes’ and survivors in the control and
treated groups was observed. After the 61-day observa-
tion period, the 33 tumor-free survivors and a group of
twenty untreated control mice of the same age and sex
were then challenged with 4.0 million Ehrlich's carcinoma
cells and observed for an additional 26 days. Only one
tumor developed in the 33 pretreated mice compared with
twenty tumors in the twenty control mice. The results
showed that repeated exposure of mice to CCRF-I cells
will protect them from subsequent challenge with the more
virulent TUC-S180 and Ehrlich's carcinoma.
The other cell line, CCRF-II, which does not require added tryptophan for growth, and which Foley and Drolet (8) showed to be more neoplastic than CCRF-I, was used for the pretreatment of mice prior to challenge. One group of mice (Chart 2) received zymosan during the treatment period in an attempt to decrease the number of tumor 'takes' or to increase the number of tumor regressions. The results (cf. Charts 2 and 3) showed that CCRF-II is less effective than CCRF-I in protecting mice from a challenge dose of the tryptophan-requiring strain, TUC-S180. Zymosan was ineffective under the conditions used. This confirms previously unpublished studies by the
was a significant difference in the amount of complement volumes of saline. The cells were centrifuged, washed mixed with varying amounts of TUC-S180 antiserum. fixed by CCRF-I and TUC-5180 antigens when they were magnesiu chloride (0.5 m@) were added to the diluent method of Bengston (1). Calcium chloride (0.15 m@i) and were checked to see that they contained equivalent nurn numbers of cells and total nitrogen per unit volume. removed from ascitic mice and immediately diluted with 5 diluted 1:800 (v/v) with saline. The TUC-S180 cells were grown in Eagle’s medium supplemented with horse nitrogen were used for the antigens, the possibility exists that the difference observed was due to a change in the surface antigens (Table 1).

Serologic studies.—Two milliliters of packed, washed cells were suspended in 3 ml of 0.9 % NaCl. Five milliliters of complete Freund’s adjuvant were added. Twenty-five hundredths of a milliliter was injected into each footpad of the rabbits. Four days later, 0.5 ml was injected S.C. into two sites. S.C. doses in two sites were given twice a week for 5 weeks. A new viable cell suspension was prepared for each treatment. C.F. antibody was present 21 days after the first treatment. The antisera used in these studies were collected about 35 days after the first treatment, tested for complement fixing activity, and lyophili- phylactoid reaction. The results suggested that the rabbit antibody present in anti-TUC-S180 serum does not bind with normal mouse tissues had been used. These data confirmed the results shown in Table 1. The amount of antibody present in anti-TUC-S180 serum does not bind the same amount of complement in the presence of equivalent numbers of CCRF-I or TUC-S180 cells.

A preliminary study of the relationship of the antigens present in the two lines of S-180 was made using the anaphylactic response in the guinea pig. Each of two guinea pigs was given 2.5 ml of rabbit anti-CCRF-I serum intraperitoneally. Forty-eight hours later each guinea pig received 50.0 million CCRF-I cells intracardially. Both animals showed evidence of anaphylaxis. Two other guinea pigs received 2.5 ml of rabbit anti-TUC-S180 serum I.P. and 48 hr. later they received 50.0 million CCRF-I cells intracardially. There was no evidence of anaphylactoid reaction. The results suggested that the rabbit appeared probable, whereas the CCRF-I cells, which had been carried in tissue culture for several years (7, 8), may have lost the antigens common to normal mouse tissue. In order to answer this question, a suspension of normal mouse cells was prepared by homogenizing the carcass of a normal mouse, except for the skin, intestinal tract, and feet, with 0.9 % saline in a Waring Blender. The cellular material was centrifuged and resuspended in saline.

One milliliter of the suspension of normal mouse tissue, equivalent to about 2.7 mg of dried tissue, was added per milliliter of reconstituted TUC-S180 antiserum and held for 20 hr. at 4°C, with occasional shaking. Treatment of the anti-TUC-S180 serum with normal mouse tissue under our conditions did not appear to influence the titer of antibody which reacted to the two antigens. Different results might have been obtained if repeated absorptions with normal mouse tissues had been used. These data confirmed the results shown in Table 1. The amount of complement in the presence of equivalent numbers of CCRF-I or TUC-S180 cells.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sensitizing Antigena</th>
<th>Challenge Cellsb</th>
<th>Resultc</th>
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<tbody>
<tr>
<td>I</td>
<td>CCRF-S180-I</td>
<td>CCRF-S180-I</td>
<td>+, +</td>
</tr>
<tr>
<td>II</td>
<td>CCRF-S180-I</td>
<td>TUC-S180</td>
<td>−, −</td>
</tr>
<tr>
<td>III</td>
<td>TUC-S180</td>
<td>CCRF-S180-I</td>
<td>+, Death</td>
</tr>
<tr>
<td>IV</td>
<td>TUC-S180</td>
<td>TUC-S180</td>
<td>Death, Death</td>
</tr>
</tbody>
</table>

* Sensitizing dose given intraperitoneally.
† Challenge dose given intracardially.
‡ + = Severe anaphylactic shock.
antiserum to TUC-S180 cells used for passive immunization contained an insufficient amount of antibody to CCRF-I cells to produce anaphylaxis.

The antigenic relationship of the two cell lines was studied using active anaphylaxis in the guinea pig. The results of Groups I and IV (Table 2) showed that the injection of either cell line, CCRF-I or TUC-S180, will produce sufficient antibody in the guinea pig to develop anaphylaxis to its antigen. Group II showed that the injection of CCRF-I cells will not produce enough antibody to develop anaphylaxis when an animal is challenged with TUC-S180 cells. This was in contrast to the immunologic studies in mice, where repeated treatment with the CCRF-I cells led to the protection of the mice against TUC-S180 cells. When TUC-S180 was used as the sensitizing antigen sufficient antibody was produced to induce anaphylaxis when these animals were challenged with CCRF-I cells. The difference in the results between Group III and the passive anaphylaxis studies was probably due to the antibody content of rabbit antiserum used and that induced in the guinea pig by the injection of the TUC-S180 cells.

DISCUSSION

Immunity to transplanted tumors has been demonstrated using homo- and heterozygous strains of rodents. The ability of inbred lines of mice and rats to produce antibodies against tumors originating within the same strain has been reported by Prehn (19), Hirsch (13), Gross (12) and Goldner (11). Goldner, using a rat ascitic tumor, 9A, which would grow as an ascitic but not as a S.C. tumor, produced immunity to the ascitic tumor by repeated S.C. injection of the tumor cells. The presence of humoral antibody was demonstrated by neutralization techniques, as well as by in vitro cytotoxic activity of the serum.

The present study shows that repeated antigenic stimulation with a nutritional variant of S-180 protects mice when they are challenged with a more virulent line of the same tumor. Protection was also noted when mice were challenged with Ehrlich’s carcinoma cells. Preliminary serologic studies indicate that the protection in the mouse studies may be due to the presence of certain common antigens. The protection demonstrated in this study is believed not to be due to nonspecific stimulation of the reticuloendothelial system, since the zymosan-treated controls developed tumors when challenged with the TUC-S180 cells (Chart 3). Further serologic studies are necessary to adequately define the antigens involved and to explain some of the discrepancies noted. Since whole cells rather than cellular extracts were used as antigens, the differences noted may reflect qualitative or quantitative changes in the cell surface proteins of the two lines of S-180. The possibility that a portion of the protective effect of pretreatment with CCRF-I cells may be due to elevated properdin levels, as suggested by Garcia and McKee (9), cannot be ruled out at this time.

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