etiocholanolone with inflammation (Fig. 1-D) but only 2 of 5 responded to lithocholic acid. Conversely, the rats responded to lithocholic acid but not to etiocholanolone (Fig. 1-A and C). The factors responsible for these variations are unknown. It is possible that the variable order of hemolytic activity exhibited by different hemolysins against erythrocytes of several species(5) may have a similar basis.

The role of the solvent vehicle in eliciting inflammation is not clear, but it seemed to be of more importance when inflammation was present elsewhere. For example, none of the injections in rats receiving neutral steroids produced inflammation, while all of the control sites in rats with lithocholic acid inflammation showed inflammatory changes (Fig. 1-C and B). Furthermore, in all species, most of the animals with inflammation at both injection sites had much more intense inflammation at the steroid site. Therefore it seems possible that in some cases the changes at the control site may actually have been secondary in some way to the intense inflammation at the adjacent steroid injection site.

Lithocholic acid, like the neutral steroid pyrogens, failed to elicit fever in animals, and therefore it does not seem possible at present to study the mechanism of steroid pyrogen activity in species other than man. It is possible, however, to study the inflammatory and cytotoxic activity of steroids in animals, and such studies may be of importance in understanding certain effects of these substances, such as their ability to produce hemolysis(6), cirrhosis of the liver(7), and other phenomena.

*Summary.* Three steroid pyrogens known to produce fever and inflammation in man were examined for inflammatory activity in rabbits, guinea pigs, hamsters, rats and dogs. Etiocholanolone, 11-ketopregnanolone and lithocholic acid are all capable of eliciting intense inflammatory reactions in experimental animals, although considerable variation in individual and species sensitivity was observed. Lithocholic acid, like the neutral steroids, failed to produce fever in rabbits, rats, dogs, cats and monkeys, thus confirming the highly specific nature of this activity in humans.

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## Influence of Peritoneal Inflammatory Exudate on Development of a Transplantable Mouse Ascites Tumor. (30111)

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It is generally known that a more or less intense inflammatory reaction with participation of infiltrating leucocytic cells frequently accompanies growth of tumors, especially transplanted tumors. The role played by this reaction in evolution of malignant growth has been considered by many authors (1-4). The purpose of this investigation was to study the influence of inflammatory exudate produced by intraperitoneal injections

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of glycogen solution on the growth of a transplantable ascites tumor.

*Materials and methods.* The dL-46 tumor used in these experiments was obtained initially by one of us (J.K.) by repeated subcutaneous injections of actinomycin "S" into an $F_1$ hybrid obtained by cross of C57BL/He male and ta albino female mice(5).

This tumor was subsequently transformed to an ascites form(6) and finally adapted easily to our stock of Swiss mice in which it was maintained by mouse to mouse passages since 1962.

Two- to 3-month-old males of inbred Swiss mice were used throughout these experiments. They were inoculated with a suspension of dL-46 cells obtained from 8-10 days tumor ascites. Cells were washed once, suspended in a medium containing Eagle's medium supplemented with 5% of decomplemented calf serum and inoculated in 0.1 ml volumes not later than 2 hours after preparation.

To produce an aseptic inflammatory exudate, mice were inoculated intraperitoneally during 3 consecutive days with 4 ml of 0.1% glycogen in physiological saline solution, as described previously(7).

*Results.* Swiss mice inoculated with standard doses of $5 \times 10^3$ dL-46 cells usually developed malignant ascites which became apparent from the 12th-15th day, progressing until the death of the animal in 20 to 40 days. The TD50 for young adult Swiss mice was of the order of $10^3$ cells per inoculum.

Mice tolerated the repeated intraperitoneal glycogen injections quite well. The inoculated mice developed an increased number of cells in the peritoneal cavity, the total wash fluid containing, on the average, 10 million cells instead of the 7 million recovered on the average from untreated mice. The percentage of macrophage type cells was also somewhat higher in the treated mice: nearly 50% as compared to 30% in the controls.

When production of malignant ascites in mice pretreated for 3 consecutive days preceding the inoculation of tumor cells by intraperitoneal injections of glycogen was compared with that in normal mice, it appeared that this treatment was effective in decreasing significantly the proportion of mice developing malignant ascites (Table I).

TABLE I. Results of Inoculation of dL-46 Tumor Cells in Normal and Glycogen Pretreated Mice.

| No. of dL-46 cells per mouse | Proportion of takes* in: Mice pretreated with glycogen for 3 days | Proportion of takes* in: Untreated mice |
|---|---|---|
| 500 | 0/10 | 3/8 |
| 1000 | 3/10 | 6/10 |
| 5000 | 0/10 | 9/10 |

* Observations 5 weeks after inoculation of tumor cells.

In another experiment mice were challenged with dL-46 cells at different times after the 3 daily routine intraperitoneal injections of glycogen. All mice in this experiment were inoculated simultaneously with the same tumor cell suspension. The most pronounced sparing effect of glycogen pretreatment appeared when dL-46 cells were inoculated 2 to 3 days after the last glycogen injection. This effect decreased progressively but lasted for a period of at least 10 days (Table II).

To explain the mechanism of the protective effect of intraperitoneal glycogen injections and, more specifically, to clarify the possible role of peritoneal macrophages in the tumor cell rejection, the following experiment was performed: Mice were treated as usual by 3 daily injections of glycogen; their peritoneal cavities were then washed thoroughly with Eagle's medium supplemented with 5% calf serum and 5 units of heparin per ml. The collected wash fluid (nearly 10 ml per mouse) was spun down and the recovered cells transferred into new mice which were then challenged with dL-46 cells. This was done in two ways: 1) mice were preinoculated with 8 million peritoneal cells and 8 hours later challenged with 5000 dL-46 cells; 2) exudate cells were mixed with dL-46 cells in the proportion of 10 exudate cells

TABLE II. Duration of Sparing Effect of Intraperitoneal Glycogen Injections.

| | Proportion of takes* with 5000 dL-46 cells in treated mice | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Untreated mice | Days after last injection of glycogen: 1 | 2 | 3 | 4 | 5 | 7 | 10 | 15 |
| 9/10 | 4/10 | 1/10 | 3/10 | 5/10 | 5/10 | 5/10 | 5/10 | 8/10 |

* Observations 3 weeks after challenge.

TABLE III. Effect of Addition or Removal of Peritoneal Macrophages on the Growth of dL-46 Ascites.

| Treatment of mice | Proportion of takes in mice challenged with: 5000 dL-46 cells | 50,000 dL-46 cells |
|---|---|---|
| Untreated | 9/10 | 10/10 |
| Glycogen treated | 5/10 | |
| " " and peritoneal cavity washed | 5/10 | |
| New mice receiving 8 million peritoneal cells 8 hr before challenge | 8/10 | |
| Inoculated with a mixture of exudate and dL-46 cells, 10:1 | | 10/10 |

to one tumor cell and the mixtures inoculated into new mice. Glycogen treated mice, whose peritoneal cavities were washed out, were used as additional controls.

Exudate cells, approximately half of them macrophages, transferred to new mice in the proportion of 10 to 1600 exudate cells for every tumor cell, failed to confer resistance against the challenge with dL-46 tumor cells. Glycogen treated mice, deprived of the bulk of the accumulated macrophages by washing of the peritoneal cavity, did not lose their relative resistance to challenge with malignant cells (Table III).

In view of these results, the question arose as to whether intraperitoneal injections of glycogen had only a local effect limited to the peritoneal cavity or had an influence on the systemic resistance of the animal against the tumor graft by means of a more general stimulation of the reticuloendothelial system (RES). To answer this question, subcutaneous inoculations of dL-46 cells were performed in control mice and in mice pretreated intraperitoneally with glycogen in the usual way. It can be seen (Table IV) that the intraperitoneal injections of glycogen had no effect on the number of tumors developing after subcutaneous inoculation of the indicated numbers of tumor cells.

The next step in our experimentation was to explore whether the presence of a peritoneal stimulation, obtained by glycogen injections, produced its anti-tumor effect in mice through a mechanism of reinforcement of the specific immunological anti-tumor or anti-homograft reaction. The following experiment was performed: Two groups of mice were immunized by intraperitoneal inoculation of $3 \times 10^5$ irradiated (5000R) dL-46 cells per animal. The "A" group contained 40 untreated Swiss mice. In the "B" group, the same number of mice were treated during the 3 days preceding the immunization by intraperitoneal injections of glycogen in the usual way. Eighteen days after the immunizing injection of the irradiated cells both groups of mice and a third group, "C," of 20 controls unimmunized mice were challenged by intraperitoneal inoculations of graded doses of dL-46 cells.

It can be seen (Table V) that one intraperitoneal injection of $3 \times 10^5$ irradiated dL-46 cells had an unequivocal immunizing effect against further challenge with relatively high doses of viable tumor cells. However, mice having a glycogen induced exudate at time of immunization had definitely no advantage over the untreated controls and did not elaborate a more intense immunity against the dL-46 cells.

*Discussion.* It is clear from these experiments that 3 daily intraperitoneal injections of glycogen solution increase significantly the resistance of mice to intraperitoneal inoculation of dL-46 ascites tumor cells. This relative resistance is evident for a period of 10 days after the last injection of glycogen but not at 15 days.

An increase of non-specific resistance to tumor grafts, especially homografts, following stimulation of the RES by BCG vaccination

TABLE IV. Results of Subcutaneous Inoculation of dL-46 Cells in Mice Pretreated Intraperitoneally with Glycogen.

| Group of mice | Proportion of subcutaneous tumors in mice inoculated with dL-46 cells with: $3 \times 10^4$ cells per inoculum | $3 \times 10^5$ cells per inoculum |
|---|---|---|
| Untreated | 5/10 | 9/9 |
| Pretreated with 3 intraperitoneal injections of glycogen | 8/10 | 10/11 |

TABLE V. Results of Immunization with Irradiated dL-46 Cells in Normal and Intraperitoneal Glycogen Pretreated Mice.

| | Proportion of takes in mice challenged with different doses of dL-46 cells: | | | |
|---|---|---|---|---|
| | $2 \times 10^4$ | $10^5$ | $5 \times 10^5$ | $2.5 \times 10^6$ |
| A. Untreated mice immunized with irradiated dL-46 cells | 0/12 | 3/10 | 7/8 | 10/10 |
| B. Intraperitoneal glycogen pretreated mice, immunized with irradiated dL-46 cells | 3/9 | 4/10 | 10/10 | 10/10 |
| C. Normal, non-immunized control mice | 10/10 | 10/10 | | |

or other means was reported by many authors (8,9).

Although the pedigree of the Japanese hybrid mouse in which the dL-46 cells originated initially is not very well determined, these cells must be considered as homografts when transferred in Swiss mice, although the host anti-homograft reaction was rather weak in this particular case since as few as 500 cells were sufficient to initiate a fatal ascites.

Since the results of the present study showed that the glycogen injections did not intensify the immune response to irradiated cells, an alternative explanation of the rejection of dL-46 cells by some of the glycogen treated mice may perhaps be based on the stimulation of less well defined, non-specific defense mechanisms linked to the activity of the RES. These mechanisms apparently do not consist simply in the destruction of tumor cells by peritoneal macrophages, since it could be shown that a mixture of peritoneal exudate cells with dL-46 cells (nearly 10:1 or 1600:1) did not influence the multiplication of these cells and the production of ascites. Moreover, removal of the bulk of macrophages by washing of the peritoneal cavity did not eliminate the induced resistance. Consequently, this temporary refractory state in some of the glycogen treated mice seems to be related to an as yet undefined "reactivity" of the peritoneal cavity rather than to the actual presence of an increased number of peritoneal macrophages. This conclusion is also supported by the fact that the total number of cells and the number of macrophages in the peritoneal cavity are only moderately increased by the glycogen injections.

Another significant point in this connection is the local character of the protection induced by the intraperitoneal glycogen treatment, illustrated by the undisturbed growth of subcutaneously inoculated dL-46 cells in the glycogen treated animals.

*Summary.* Three daily peritoneal injections of 1:1000 glycogen solution induced in mice, during at least 10 days, a state of relative resistance to intraperitoneal but not subcutaneous inoculation of homotransplantable dL-46 ascites tumor cells. Removal of excess macrophages by peritoneal washing or their transfer to untreated mice did not influence the development of dL-46 ascites. Furthermore, elaboration of specific immune reaction by intraperitoneal inoculation of irradiated cells was not enhanced in glycogen treated mice.

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