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IMMUNIZATION STUDIES WITH EHRLICH MOUSE ASCITES TUMOR

by

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M.B., B.S., King Georges Medical College, Lucknow, INDIA. 1958.

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for the degree of

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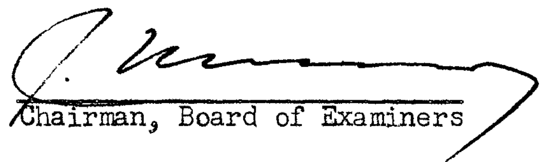

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CHAPTER I

INTRODUCTION

Hesse, in 1927 (1), was perhaps the first to show that Flexner-Jobling rat carcinoma could be transmitted by the ascitic fluid that developed during the growth of the tumor. Koch (2), one year later, confirmed this finding and also showed that the tumor developed better when injected intraperitoneally, and that the number of tumor cells in the ascitic fluid increased with time. In 1932, Lowenthal and Jahn (3), Koch's collaborators, obtained on injection of Ehrlich mouse carcinoma, an ascites-producing tumor instead of the usual solid tumor. This ascites tumor could be transplanted successfully through intraperitoneal injection of the ascitic fluid. Thus Lowenthal and Jahn (3) established a new line of the Ehrlich carcinoma, which they called the ascites tumor. This ascites tumor found wide use in experimental studies in Germany soon after it was discovered (4, 5, 6, 7), and in recent years it has been widely employed for other studies. Lettre (5), for example, used it to study chemotherapeutic agents, Klein and co-workers (8) studied its chemical composition, and Warburg and Hiepler (6) studied its metabolism. Hauschka and Levan (9) and Hauschka (10) recognized the extraordinary significance of this ascites tumor as a convenient material for the study of chromosomes, cell morphology and tumor transplantation immunity.

In 1948, Klein and co-workers (11) converted a solid line of Ehrlich mouse carcinoma into the ascitic form and named it the

tetraploid line of Ehrlich mouse ascites carcinoma. Since then a wide variety of transplantable tumors have been converted into the ascitic form. Interesting types of ascites tumor have been reported by Craigie (12), Makino (13), Tanaka (14), Takeda (15), Shelton (16), Isaka (17), Bather (18), Williams (19), and Fekete and Griffen (20).

The general characteristics of the Ehrlich mouse ascites tumor have been studied by Klein and co-workers (21). After an animal is given an intraperitoneal injection of the tumor fluid, it develops a progressive ascites. The time required for the ascites to become manifest varies inversely with the number of cells in the inoculum. With the usual dose of 0.1 ml of the whole fluid (approximately 9×10^6 cells), it takes 6 to 8 days for the ascites to become grossly noticeable. With lower doses it may take as long as twenty days before any ascites is recognizable, and if the dose is much lower, ascites may not be produced at all. Instead, a solid implantation and growth occur on the omentum or some other part of the peritoneal membrane (21).

The morphology of the Ehrlich ascites tumor cells has been studied by phase microscopy, ordinary light microscopy (22) and electron microscopy (23). Under the phase microscope, the tumor cells display fine hair-like pseudopodia averaging about 1 micron in length, a well defined nucleus with irregularly clumped chromatin, and the cytoplasm. The cytoplasm contains two readily distinguishable components: 1) the mitochondria that appear as short dark rodlets and 2) some brilliantly refractile globules that contain large amounts of

lipid (23). In 1958, Hirono and Iwao (24) reported that the living Ehrlich ascites tumor cells when observed in hanging drop preparations, after incubation at 37° C for 1 hour, display ameboid movement and pseudopod formation. The average measured motility of the cells was about 0.9 microns per minute.

Studies with stained preparations (25, 26, 27) have shown that the nucleus contains several prominent nucleoli. Inside many of these nucleoli there are fine vacuoles. In addition to the nucleoli and the chromatin, the nucleus also contains finely granular parachromatin (26) which stains acidophilic. In old tumors, the chromatin and the parachromatin of an occasional cell are marginated and 1 or more acidophilic bodies are formed. The nature of these bodies is made clear by the use of toluidine-blue-molybdate stain (27), which stains the desoxynucleoprotein content of the cell. By this method the desoxynucleoprotein of the parachromatin stains purple and that of the nucleolus green. Many of the nucleoli contain metachromatic ribonucleoprotein bodies or nucleolini (28), which appear to correspond to the vacuoles seen in other preparations. Under the microscope it can be seen that the large inclusions consist of a homogeneous green-staining center, derived from the nucleolus, covered by purple parachromatin granules. In degenerating cells smaller inclusions appear to be entirely formed by condensation of the parachromatin. A study of the old ascites tumor cells reveals that these cells eventually degenerate and disintegrate, going through the conventionally recognized processes of pyknosis, karyorrhexis and karyolysis, after

which the fragments are digested by the macrophages.

The cytoplasm of the Ehrlich ascites tumor cell contains 3 types of lipid particles (25). Some of these particles are large droplets which stain red with Nile-blue sulfate. These are especially predominant in older degenerating cells and cells that are infected with viruses (29, 30, 31). The second type of lipid droplets consists of an amorphous, acidic, Schiff-periodic acid-positive material found chiefly in the perinuclear zone of the cytoplasm. These granules consist mainly of phospholipids (32), and are found in the region where the golgi apparatus is located (22). Like the golgi apparatus, these phospholipid granules also are stained by the Kolatchew golgi apparatus staining technic (33). The third type of these lipid particles in the cell consists of the mitochondria. These are more regular in shape and are distributed more evenly than the former particles in the cytoplasm (34). In the degenerating cells of the old tumors, neither the mitochondria nor the golgi apparatus are altered significantly except during the final stage of disintegration of the cell.

Studies of the cellular component of the ascites at different phases of growth of the tumor, have shown that the cells consist mainly of tumor cells, but red blood cells and inflammatory cells are also present. The relative proportion of the red cells varies. Some fluids are grossly hemorrhagic while others are relatively clear. The red blood cells present in the fluid display poikilocytosis and hypochromesia (35). Fair proportion of these are also nucleated. The

proportion of inflammatory cells varies with the individual animal and with the age of the tumor. Generally in early stages of growth the percentage of non-tumor cells has been found to be inversely related to the number of tumor cells in the inoculum (21). A variation in the physiological ages of the inocula has little or no effect on cell count. The latent period (i.e., the period preceding the actual increase in the cell count) is prolonged when older inocula are used. Klein and co-workers (21) investigated the tumor growth using 3-, 6-, and 16-day-old fluid as inocula. They collected the fluid after a definite period, rinsed the peritoneal cavity twice with saline and determined the total volume of fluid and number of cells per animal. Thus they were able to determine the various phases of growth of the Ehrlich mouse ascites tumor. These phases are: 1) the latent phase, 2) the phase of logarithmic growth, 3) the stationary phase in which more fluid than cells is formed and 4) the regression phase during which the dead cells predominate. They reported that with inocula of various ages, the growth curves were more or less parallel, and the difference between regression phases was not at all marked. On the basis of these observations, Klein concluded that the physiological age of the inoculum has no discernible effect on the proliferation of the tumor cells.

The tumor cell population consists of both living and dead tumor cells. They can be differentiated by staining a wet smear of fresh

fluid with nigrosin (36). The living cells take up the vital stain and are consequently stained black. The dead cells remain unstained. The tumor cells have been counted in different ways by different workers. Hemocytometric counting chambers have been employed most successfully and conveniently (21). An ascitocrit, a modification of a hematocrit, is the next most widely employed tool for this purpose (37). Klein and co-workers using the hemocytometer have reported the tumor cell count of the ascitic fluid at different stages of growth of the tumor (21). These workers found that after a latent period of about 48 hours, the cell count started rising more or less logarithmically till it reached the peak on or about the 9th day. The inflammatory cells were a predominant feature of the ascitic fluid during the latent period. From the 9th to the 11th day the cell count was relatively stationary. Since the amount of fluid produced was very high at this stage, the cells were diluted and the count was low per unit volume (about 1×10^8 cells per ml) as compared to the cell count at the peak of the growth curve ($2\text{-}3 \times 10^8$ per ml). After the 11th day the phase of regression ensued where the proportion of dead cells increased (21).

The fluid component of the ascites increased progressively as the infection proceeded. When 0.1 ml of the whole fluid was used as inoculum, the fluid varied from 5 to 8 ml on the 6th day to 12 to 15 ml on the 16th day. The protein content of this ascitic fluid was lower than that of serum from tumor-bearing mice (3.1 gm % in fluid

and 4.9 gm % in serum). The serum from normal mice contains 6.7 gm of protein per 100 ml. The albumin content of the ascitic fluid was especially low (0.9 gm %). The concentration of alpha- and gamma-globulins was also lower when compared with the serum (38). However, the beta-globulin level was higher (1.1 gm %) in the ascitic fluid than in the sera of mice bearing the tumor (0.6 gm%). The properdin titer of ascitic fluid was never more than 4.0 units per ml, whereas the serum of tumor-bearing mice contained 10 to 40 units of properdin per ml.

The most obvious effect of tumor growth on the host is the production of ascites. This ascites formation leads to respiratory embarrassment, toxemia, hepatic dysfunction, renal insufficiency and finally death. In 1933, Putnoky (35) followed the changes in the peripheral blood in rats after injecting the tumor intraperitoneally. He noticed a rise in polymorphonuclear count accompanied by a simultaneous fall in the number of the lymphocytes. Monocytes and eosinophils also decreased in number. The platelet count showed a transient rise. Anemia was a marked feature and the red blood cells displayed anisocytosis, poikilocytosis and polychromatophilia. Nucleated red cells and reticulocytes were abundant. Coagulability of the blood was unaffected. Putnoky (35) also reported that these changes were reproducible with autolysates, emulsions and ethereal extracts of necrotic tumor cells.

The literature on tumor immunity is so extensive that even as early as 1916, Tyzzer (39) stated that "It is quite impossible to present within reasonable space, a comprehensive review of all

investigations done on tumor-immunity." A second review by Waglom in 1929 contained over six hundred references (40).

Specific immunity to transplantable tumors is the reaction of an organism to a foreign cell. The degree of this reaction or immunity depends on the foreign-ness of the tumor cells. Much work has been done in this field. Caspari in 1929 (41), introduced the concept of "necrohormones" which he thought were produced as a result of degeneration of the tumor and caused the development of a general immunity. Lumsden, (42) in 1931, went to the extent of saying that "There is no antibody whose existence is more proved than that of these anti-malignant-cell antibodies." He described five types of antibodies against tumor cells: 1) anti-species antibody, 2) homologous cytotoxins, 3) normal hetero-cytotoxins, 4) autocytotoxins, and 5) cytotoxins for malignant cells present only in animals with tumors. In 1937, however, Phelps (43) showed that the antibodies of the so-called anti-sera of Lumsden had no specific action on the cancer cells and were not peculiar to the malignant cells. He described them as isoantibodies in response to foreign though homologous cells that were injected.

Gorer (44, 45) introduced the idea that genes were responsible for isoantigenic differences and showed that certain important isoantigens were shared by mouse erythrocytes, normal fixed tissues

and the tumor cells. Hauschka (10) postulated that successful transplantation of a graft depended on the simultaneous presence of dominant genes, in the graft as well as in the host. He also said that this was true for normal as well as for neoplastic tissues. If a tumor graft carries one or more dominant loci functioning in the direction of elaboration of cellular antigens, and the host has inactive or immunologically different alleles of these genes, the implant evokes antibodies against itself and usually regresses. If on the other hand the pertinent antigenic alleles are dominant in both the tumor and the host, no immune reaction ensues, and the tumor transplant continues to grow.

It has been shown that most of the malignant cells contain various antigens (46, 47, 48, 49, 50), some of which are common to normal tissues. Various reports have appeared regarding the demonstration of specific neutralizing and/or agglutinating antibodies against different tumors. Neutralizing antibodies against Rous Sarcoma were described in the sera of recovered chickens by Kabat and coworkers (51). Kidd demonstrated neutralizing antibodies against Brown-Pearce carcinoma (52) and Shope papilloma (53). Bennison (54) and Imagawa (55) demonstrated antibodies against Bittner milk agent for mammary carcinoma. Flax (80) and Horn (81) reported the successful neutralization of the carcinogenic property of Ehrlich ascites tumor cells by incubating them with rabbit antiserum. Flax, however, could not demonstrate any _in vitro_ neutralization without the addition of

guinea pig complement to the antiserum. The gamma-globulin fraction of this antiserum when combined with guinea pig complement could also neutralize the tumor (80). Treatment of this gamma-globulin-complement complex at 56° C for 30 min destroyed its neutralizing power. Horn (81) was able to demonstrate neutralization of the tumor without the addition of complement. Flax also observed that the average survival period of the tumor-bearing mice was almost doubled when they were treated with the antiserum or gamma-globulin fraction of this antiserum after challenge. Recently Sukhorukikh (82, 83, 84) demonstrated a definite cytotoxic effect of agglutinating antiserum on the tumor cells. The same author (83) also demonstrated the inhibition of mitotic activity of the tumor cells after incubation with the agglutinating antiserum. Normal rabbit serum and antisera prepared against liver and spleen from normal mice, failed to produce this inhibition.

Snell and his associates (56) showed that dead tissue or tissue products could be used as active immunizing agents against transplantable tumors. Tizzoni and co-workers (57), in 1930, used malignant cells killed with formalin and phenol to immunize mice against an adeno-carcinoma (SE81). Bittman and Goldfeder in 1929 (58) prepared antisera against Ehrlich mouse carcinoma and demonstrated that mice injected with this antiserum before tumor transplant were not protected, while if the antiserum was given after the tumor, there was a slight inhibitory effect. Costero in 1930 (59) made attempts to immunize mice against Ehrlich mouse carcinoma by injecting intravenously autolysates

of muscles from cancerous mice but was not successful. Schabad in 1939 (60) reported that addition of splenic tissue from normal mice inhibited the growth of Ehrlich mouse carcinoma, whereas spleen from cancerous mice lacked this property. Lund (61) in 1958, was successful in immunizing rats against Yoshida sarcoma by intracecal injection of tumor cells. Recently Donaldson and Mitchell (62) immunized 90% of mice against Ehrlich ascites tumor by using tumor cells killed with x-rays, and Donaldson and North (63) immunized mice against this tumor by using tumor cells killed with nitrogen-mustard.

Before closing this chapter, the subject of non-specific immunity to tumors will be briefly reviewed. It is known that administration of neutral polysaccharides of diverse plant, animal and microbial origins to experimental animals profoundly affects the resistance to bacterial infection and growth of tumor transplants. Large doses of zymosan or bacterial polysaccharides transiently decrease the resistance of mice to gram-negative bacterial infection (64, 65) and cause increased growth and lethality of Sarcoma 180 in mice (66), human epidermoid carcinoma in rats (67), and human intestinal adenocarcinoma in rats (68). Conversely, small doses of polysaccharides increase the resistance of mice to infection (65, 69, 70, 71, 72, 73) and increase the rejection of Sarcoma 180 (66, 74) and Sarcoma 37 in mice. Lack of cross-antigenicity between zymosan and these pathogens indicates that these effects are the result of an initial depression followed by stimulation of non-specific host defense mechanisms. The effect of

polysaccharides on susceptibility to infection and tumor growth varies inversely as its effect on serum properdin levels (70, 71, 74), suggesting that properdin, or another defense mechanism which fluctuates simultaneously with properdin, may be involved in tumor rejection mechanism. Kidd (76), Ainis (77) and Herbut (78) have demonstrated in guinea pig serum a non-specific humoral factor, probably different from complement and properdin, which inhibits the growth of a rat lymphosarcoma.

To summarize the issue of immunity and cancer, Southam (79) may be quoted: "In spite of the complete absence of proof, the existence of so many suggestive bits of clinical and experimental data sustains the working hypothesis that cancer is not a wholly autonomous growth but may be susceptible to restraint by the host mechanisms."

CHAPTER II

STATEMENT OF PROBLEM

From the review of the literature, it appeared reasonable to assume that tumor transplants are influenced by the host defense mechanisms which could be modified by specific and non-specific means. With this assumption in mind, it was decided to study: 1) active immunization of mice by the use of Ehrlich ascites tumor cells treated with different physical and chemical agents, 2) passive immunization by the use of Ehrlich-ascites-tumor-cell-agglutinating rabbit antiserum, and 3) non-specific immunization induced by *Bordetella pertussis* cells, zymosan, *Escherichia coli* endotoxin and Freund's incomplete Bacto-adjuvant.

CHAPTER III

MATERIALS AND METHODS

MATERIALS:

Tumor: A line of the ascitic form of Ehrlich ascites tumor was obtained from Dr. P. C. Rajam of the University of Michigan on May 9, 1959, and was maintained in the Stella Duncan Laboratory, Montana State University, through weekly intraperitoneal transfers in female mice.

Mice: In most of the experiments, 5- to 7-week old mice of the CFW (Swiss Webster) strain purchased from Carworth Farms, New City, New York, were used. In some experiments, the strain of white mice kept at the Rocky Mountain Laboratory, Hamilton, Montana, was used.

Rabbits: Male rabbits weighing between 4 and 6 pounds were used for preparing the antiserum. These rabbits were of undetermined strain, and were purchased from the Dry's Rabbit Farm, Veradale, Washington.

Physiological saline: 0.85% sodium chloride in distilled water is referred to as physiological saline.

Formalin: 36.3% formalin solution, lot No. 545228, obtained from the Fisher Scientific Company was used.

Phenol: Crystalline phenol, lot No. 90851, obtained from the J. T. Baker Company was used.

Merthiolate: 1:1,000 merthiolate (thimerosal), lot No. 6133-738850, containing 0.1% monoethanolamine and obtained from Eli Lilly Company was used.

Freund's Adjuvant: 10.0 ml ampoules of Freund's Incomplete Bacto-adjuvant, lot No. 0639-60, control 443805 purchased from the Difco Laboratories were used.

Bordetella pertussis Cells: Cells were grown in the liquid medium of Verwey *et al.* (88), and were killed by the addition of 0.01% merthiolate. These merthiolate-killed cells were kept in a suspension containing 700 billion cells per ml in saline with 0.01% merthiolate.

Escherichia coli Lipopolysaccharide: Batch No. L583-362, 10-1, obtained from Merck Sharp and Dohme Company through the courtesy of Dr. O. Ganley was used.

Zymosan: Lot No. 71313, obtained from the Fleischmann Laboratories, Betts Avenue, Stanford, Connecticut, was used.

METHODS:

Collection of Fluid: From the growth curves obtained in mice inoculated with 0.2 ml of Ehrlich mouse ascites tumor fluid, it can be said that the maximum number of cells (about 1×10^8 per ml) is obtained around the 8th day after the inoculation. At this stage, the number of dead tumor cells and inflammatory cells is at its lowest point (21). For these reasons, the tumor fluid for all experimental purposes (unless stated otherwise) was collected by tapping the mice on the 8th day after the tumor inoculation. The tapping was done with an 18 gauge sterile needle inserted in the region of the left iliac fossa. The fluid was allowed to flow out into a test tube. On many occasions the needle was removed and the fluid allowed to flow out of

the puncture wound. The fluid thus obtained was stored in the refrigerator or used immediately. In no case was a sample of fluid stored for more than 18 hours, as it has been shown that after 30 hours in the refrigerator, most of the cells are dead.

Washing the Tumor Cells: Tumor cells were obtained reasonably free from fibrin clots and necrotic debris by centrifuging the whole fluid at a rate of 2,000 rpm for 5 min at 6° C in a model S-S Servall centrifuge. The turbid supernatant was pipetted off, mixed with an equal volume of physiological saline, and centrifuged at 5,000 rpm for 5 min. The sedimented cells were resuspended in saline and mixed thoroughly. Then they were spun down again. This was repeated 5 times or until the cells ceased to form sticky clumps (85). Cells properly washed went into suspension rather easily.

Standardization of Cell Suspensions: Serial tenfold dilutions of the cells were made in saline by adding 0.5 ml of suspension to 4.5 ml of saline. The third and fourth dilutions were counted in a Neubauer-type Hemocytometer counting chamber. The cell content was calculated as follows:

$$\frac{\text{Total cell count in the four chambers x 10}}{4} = \text{No. of cells per cu mm}$$

Suspensions for Challenge: The fluid for this purpose was usually obtained from a female CFW mouse that had received the tumor 8 to 10 days previously. Dilutions were made in saline to give a final cell count of 20,000 per ml. Four to six thousand cells (0.2 to 0.3 ml) were injected intraperitoneally. The suspension was injected within

half an hour after making the dilutions in order to avoid any serious injurious effect of saline on the tumor cells. This dosage of 4,000 to 6,000 cells was chosen because it was shown that the ascites became manifest by the 20th day after this challenge. With lower doses the incidence of the tumor in ascitic form was lower and ascites became apparent only after a longer period of time (35 to 40 days).

Treatment with formalin: Cells were separated from 5.0 ml of fluid and washed 3 times with saline. Then they were resuspended in saline to a final concentration of 5×10^7 cells per ml. To 4.0 ml of this suspension contained in a 25 ml pyrex glass beaker were added 0.04 ml of formalin and 16.0 ml saline. The suspension was thoroughly mixed and allowed to stand at room temperature for 2 hours with occasional manual agitation, and then kept in the refrigerator for 16 hours. At the end of this period this cell suspension was used to vaccinate mice. Freshly formalinized cells were used for each vaccination.

Treatment with phenol: Saline-washed cell suspensions containing 5×10^7 cells per ml were made as for the preparation of formalinized cells. The cells from 20.0 ml of this suspension were separated by centrifugation and resuspended in 20.0 ml of 0.8% phenol in a 50 ml pyrex glass beaker. This suspension was thoroughly mixed and allowed to stand at room temperature for 1 hour and then in the refrigerator for 18 hours. At the end of this time, the cells were separated and washed twice with saline to remove excess phenol, and then resuspended

in 20.0 ml of saline. This suspension was used to vaccinate mice. Freshly treated cells were used for each vaccination.

Treatment with merthiolate: A washed cell suspension containing 5×10^7 cells per ml was prepared as above. Three ml of this suspension were added to a 25 ml beaker containing 5.0 ml of saline and 2.0 ml of 1:1000 merthiolate. This suspension was allowed to stand at room temperature for 2 hours with frequent shaking, and then kept in the refrigerator for 16 hours. At the end of this period, the suspension was used to vaccinate mice. Freshly prepared merthiolate-treated cells were used for each vaccination.

Preparation of disrupted cells: Approximately 4 gm of No. 12 Cataphote glass beads and 5.0 ml of a saline suspension of cells containing 5×10^7 cells per ml were placed in a Mickle reed and shaken in a Mickle disintegrator for 4 min. This was repeated 4 times with 5 to 10 min intervals between each treatment. Samples were examined under the microscope to determine the degree of disruption. If less than 80% of the cells were disrupted, the mixture was shaken once more for 4 min. Then the glass beads were allowed to settle and the broken cell suspension was pipetted off. When only cell extract was desired, this suspension kept at 6° C was centrifuged at 8,000 rpm for 10 min and the supernatant was decanted and again similarly centrifuged to remove the cell debris.

Irradiation with ultraviolet light: A G15-8T model ultraviolet lamp (2,537 Å) purchased from the General Electric Company was used.

No exact measure of the ultraviolet light intensity emitted from this lamp was made and for this reason it is not possible to specify in exact terms the dose of ultraviolet light administered to the tumor cells. To vary the dose, only the time of exposure was varied. The procedure used was as follows:

Two and one-half ml of the tumor fluid were diluted ten times by adding 22.5 ml saline. Ten ml of this diluted fluid were placed in a glass petri dish 9.0 cm in diameter. After allowing the lamp to warm up for 15 min, the dish containing the fluid was placed on a platform at a distance of 30 cm below the center of the lamp. This platform was mechanically rocked to keep the fluid under constant agitation. Samples of fluid were irradiated for 10 and 15 min, and then injected into mice within 15 min after irradiation. The energy emitted by the lamp under the specified conditions was approximately 32.3 ergs/mm^2/sec (86). Therefore, the energy derived from 10 min irradiation was approximately 10 x 60 x 32.3 or 19,380 ergs/mm^2, and that from 15 min irradiation, 20,070 ergs/mm^2.

Immunization of rabbits: Six rabbits were divided into 2 groups of 3 each; group I received intact tumor cells and group II received disrupted cells. Thirty ml of blood was collected from each rabbit before starting the immunizing injections. Then they were given subcutaneous injections of 2.0 ml of the immunizing material (washed cells in saline with 9 x 10^7 cells per ml or 2.0 ml of the broken-cell suspension). These injections were given at three-day intervals for a

total of 9 injections. Thereafter the rabbits were trial-bled from the marginal ear vein at three-day intervals, and the serum agglutinin titer determined. The titer reached its peak on the 26th day after the last injection and then it started to decrease. On the 28th day the rabbits were given another subcutaneous dose of 2.0 ml of unbroken washed cell suspension containing 9 x 10^7 cells per ml. The agglutinin titer was again followed. Fifteen days after the booster dose, when the agglutinin titer was high, the rabbits were bled to death from the heart. The serum was obtained and stored in the frozen state (-20° C).

Agglutinin titration: Serial two-fold dilutions of the antiserum were made in 1:100 normal rabbit serum in saline. Saline and 1:100 normal rabbit serum in saline were used as controls. To 0.5 ml of the respective dilutions, 0.5 ml of a suspension of tumor cells (washed four times) in saline containing 4 x 10^5 cells per ml was added. The tubes were thoroughly shaken and allowed to stand at room temperature for 2 hours and then overnight in the refrigerator. The results were then recorded as -, +, ++, +++, & ++++ depending on the degree of clumping of the tumor cells noticed after shaking the tubes gently. In reading this agglutination every tube was compared with the normal rabbit serum and the saline controls.

Technic of absorption of the rabbit antiserum:

A. Specific absorption with washed tumor cells: Tumor cells obtained from 5.0 ml of ascites fluid were washed 4 times with saline

and then sedimented. These sedimented cells were then added to 12.0 ml of rabbit antiserum (titer 1:2560), mixed thoroughly and allowed to stand in the refrigerator. Twenty-four hours later, the antiserum was separated by centrifuging at 12,000 rpm for 15 min. Fresh tumor cells obtained from 5.0 ml of ascites fluid were washed 4 times with saline, sedimented, and then added to this antiserum. This was repeated and the agglutinin titer determined after each absorption. At the end of the 7th absorption, only slight and doubtful agglutination was observed in 1:2 and 1:4 dilutions. This antiserum was then filtered through a No. 12 Mandler bacterial filter and stored in the frozen state (-20° C).

B. Non-specific absorption with normal mouse liver and spleen: Liver and spleen obtained from 3 normal CFW female mice (about 5-week old) were washed twice with saline and then cut into small pieces. These pieces were washed once with saline and then sedimented from saline suspension by centrifuging at 5,000 rpm for 15 min. These sedimented pieces were then added to 12.0 ml of rabbit antiserum (titer 1:1,280) and mixed thoroughly. This suspension was allowed to stand in the refrigerator. After 24 hours, the antiserum was separated by centrifuging at 5,000 rpm for 20 min and fresh liver and spleen pieces (washed with saline) obtained from 3 female CFW mice were added to it. This suspension was allowed to stand in the refrigerator for 24 hours and at the end of this period the antiserum was separated. The absorption was repeated 7 times. At the end of the 7th absorption, the agglutinin titer was found to be 1:512. The dilutions showing maximum

agglutination (4+) were found to be 1:160 and 1:320. This absorbed antiserum was filtered through a No. 12 Mandler bacterial filter and stored in the frozen state (-20° C).

CHAPTER IV

EXPERIMENTAL RESULTS

I. Preliminary studies:

Experiment 1. Minimum number of tumor cells necessary to produce ascites in female CFW mice. Two dilutions of ascites fluid were made in saline, one containing 1,000 and the other 20,000 cells per ml. These suspensions were injected into mice in varied doses as shown in Table I. Mice in group I were injected with 0.2 ml of the cell suspension containing 1,000 cells per ml, or 200 tumor cells per mouse. All other groups were injected with varied amounts of the suspension containing 20,000 cells per ml. It is evident from the results in Table I that the time required by the tumor to manifest itself in the form of recognizable ascites varied inversely with the number of tumor cells in the transplant. With a dose of 200 cells ascites was noticeable on the 28th day in only 3 of 10 mice injected. With a transplant of 16,000 tumor cells, however, ascites was noticed in a few mice by the 6th day after the challenge, and 10 of 10 mice developed ascites by the 15th day.

Experiment 2. Comparative susceptibility of female mice of the CFW and CF1 strains. Tumor fluid obtained separately from CFW and CF1 mice was used for challenging the two breeds of mice. Cell suspensions with varied cell concentrations were injected into groups of 5 mice as shown in Table II. The incidence of ascites was recorded on the 15th and 28th day after the challenge. From the results in Table II,

TABLE I

MINIMUM NUMBER OF TUMOR CELLS AND ASCITES

Group	No. of cells	Positives* on (days) 6th	8th	10th	15th	20th	28th	31st
I	200	-	-	-	-	-	3/10	7/10
II	1000	-	-	-	-	-	4/5	5/5
III	2000	-	-	-	-	9/10	10/10	10/10
IV	4000	-	-	-	10/10	10/10	10/10	10/10
V	8000	-	-	3/10	9/10	9/10	9/10	9/10
VI	16000	1/10	3/10	4/10	10/10	10/10	10/10	10/10

* Mice that showed growth of tumor in the form of obvious ascites are, hereafter, mentioned as "Positives."

TABLE II

COMPARATIVE SUSCEPTIBILITY OF CF1 AND CFW MICE

Group	Challenge (No. of cells in thousands)	CF1	CFW
		No. of positives on 28th day	
I	1	5/5	4/5
II	2	5/5	4/5
III	4	5/5	5/5
IV	8	5/5	5/5

there appears to be no marked difference in the susceptibility of the two strains of mice. However, in general, the CF1 mice appeared to be slightly more susceptible than the CFW strain.

Experiment 3. Growth curve of the Ehrlich ascites tumor and differential blood count on the tumorous mice. Seventy female Hamilton mice, each about 5- to 7-week old, were injected with 0.2 ml of the ascites fluid obtained from female Hamilton mice in which the tumor was 10 days old. Groups of 5 mice were bled from the infra-orbital sinus on the desired day and smears made. These smears were stained with Wright's stain and differential white-blood-cell counts performed. After the fourth day of challenge when the ascites became manifest, 5 mice were randomly selected daily, tapped for ascitic fluid, then bled and discarded. Daily cell counts were performed on the tumor fluid thus obtained. The averages of the cell counts of fluids obtained from 5 different mice at the various time intervals investigated were plotted (Fig. 1). Differential white-blood-cell counts were performed on each of five smears and the results were averaged and plotted (Fig. 2).

It is evident from Fig. 1 that the tumor cell count of the ascitic fluid per unit volume was highest from 4 to 7 days after the tumor transplantation. At this time the cell count was found to be around 15-20 x 10^7 cells per ml. Thereafter, the cell count per volume decreased progressively until about the 14th day when it was as low as 7 x 10^7 cells per ml. The ascites, however, progressed and the

FIGURE 1.

T

TUMOR CELL COUNT OF THE ASCITES FLUID PER UNIT VOLUME

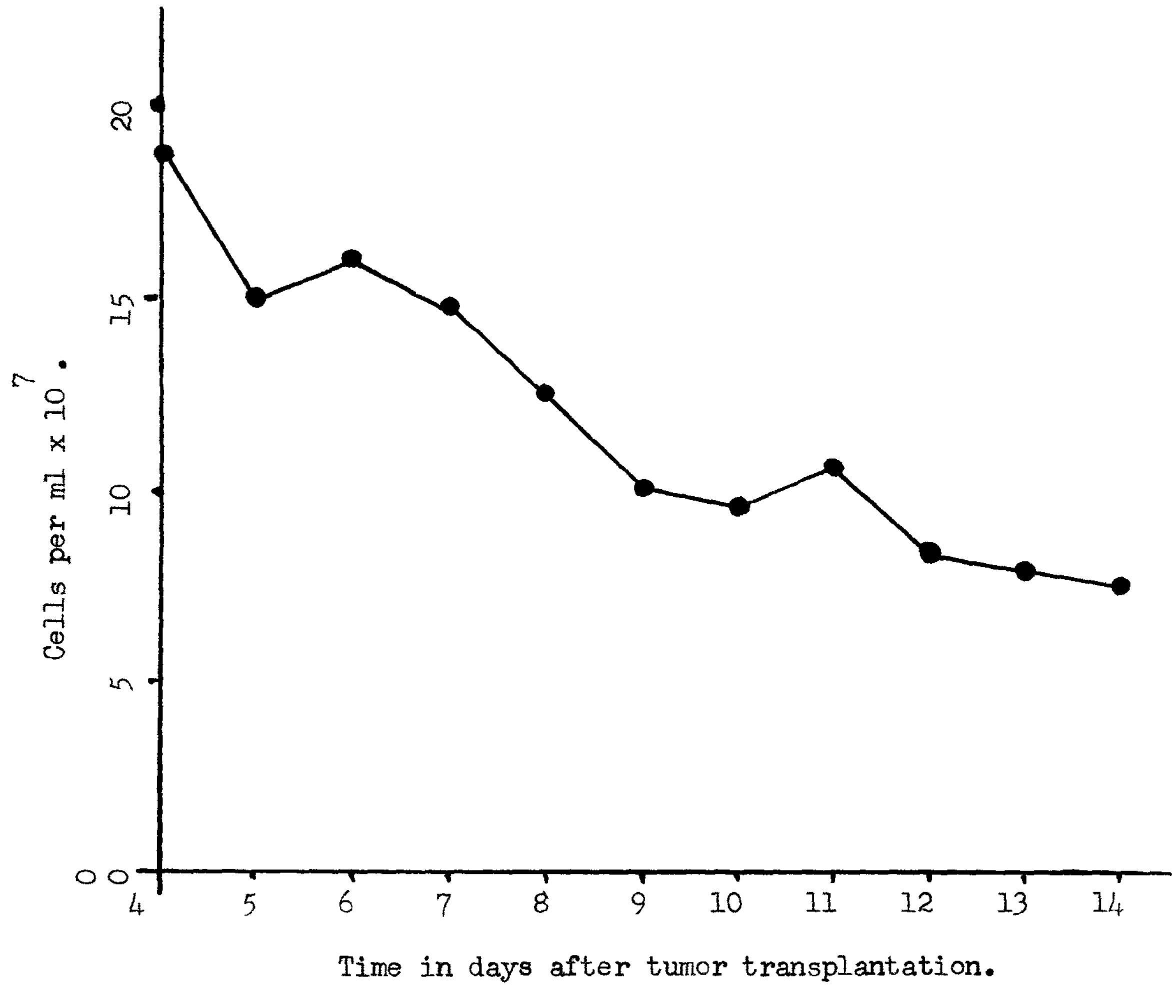
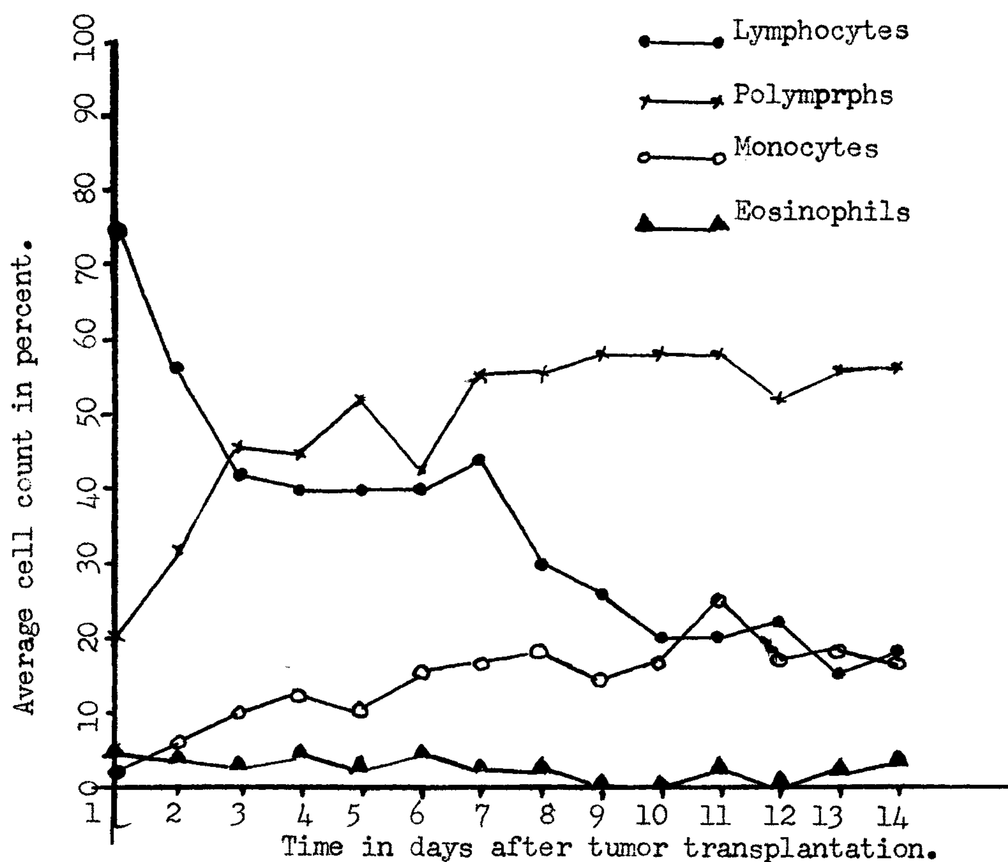


FIGURE 2.

DIFFERENTIAL W.B.C. COUNT ON BLOOD SMEARS OF TUMOROUS MICE



abdomen became more and more distended, till about the 18th to 20th day when the animals died.

The differential blood count (Fig. 2) showed a progressive rise in the neutrophils and a progressive decrease in lymphocytes. Eosinophils showed no noticeable change. The large monocytes increased gradually, reaching the highest percentage (20%) on the 11th or 12th day.

II. Active specific immunization:

Experiment 4. Lowest concentration of formalin required to kill the tumor cells. A tumor cell suspension with a cell count of 5×10^5 cells per ml was mixed with 2.0% formalin in saline, and saline in different proportions to obtain the desired concentration, as shown in Table III. Then the mixture was allowed to stand at room temperature for 1 hour. After this period, it was kept in the refrigerator for 16 hours. At the end of 16 hours, it was injected into female Hamilton mice in doses of 0.2 ml per mouse. An untreated cell suspension with the same cell count was used as control. The number of mice that developed ascites was recorded on the 15th day after the injection. The results in Table III show that a concentration of formalin between 0.25 and 0.125% was required to kill the tumor cells. From these results it was decided to use 0.2% formalin solution to prepare vaccines.

Experiment 5. Immunization of mice with formalin-killed cells. Formalin-treated cells were prepared as described in the section on materials and methods, and injected intraperitoneally in female CFW mice in 0.2 ml doses. Three immunizing injections were given at four-

TABLE III

EFFECT OF FORMALIN ON THE TUMOR CELLS

Formalin 2%	Cell suspension	Saline	Final concentration	Positives / total mice
2.0 ml	2.0 ml	-	1%	0/4
1.0 ml	2.0 ml	1.0 ml	0.5%	0/4
1.0 ml	2.0 ml	5.0 ml	0.25%	0/4
0.5 ml	1.0 ml	6.5 ml	0.125%	2/4
Controls	cells only			4/4

day intervals. Then the mice were divided into 3 groups. Group I was challenged 6 days after the last injection, group II, 10 days, and group III, 14 days after the last vaccination. The challenging dose consisted of 5,000 tumor cells. Observations as recorded on the 20th day after challenge are shown in Table IV. The results show that the induced immunity increased with time after the last vaccination. It was most marked on the 14th day after the last vaccination, when only 19.8% of the mice developed the tumor as compared to 72.5 and 42.5% on the 6th and 10th day, respectively.

Experiment 6. Lowest concentration of phenol required to kill the tumor cells. A tumor cell suspension with cell count of 5×10^5 per ml was prepared and mixed with varying amounts of 1% phenol in saline, and plain saline in order to obtain the desired concentration of phenol according to the protocol in Table V. The suspension was kept at room temperature for one hour and in the refrigerator for 16 hours. At the end of this period, the cells were separated from the phenol solution by centrifugation at 2,000 rpm for 5 min and then washed once with saline to remove the excess phenol. The sedimented cells were resuspended in saline. Two-tenths ml of this suspension was injected intraperitoneally into each mouse. Washed tumor cells not treated with phenol were used as controls. The results indicated that the lowest concentration of phenol that was required to kill the tumor cells under the specified conditions was between 0.5 and 0.8%. Therefore, 0.8% phenol was employed to prepare the vaccines.

Experiment 7. Immunization of mice with phenol-killed tumor cells. Four intraperitoneal injections of phenol-killed vaccine were given at

TABLE IV

IMMUNIZATION WITH FORMALIN-KILLED CELLS

Group	No. of vaccn.	Time of challenge after last vaccn.	Positives / total mice	% Positives
I	3	6 days	29/40	72.5%
Ia control	-	6 days	5/5	100.0%
II	3	10 days	17/40	42.5%
IIa control	-	10 days	4/5	80.0%
III	3	14 days	6/30	19.8%
IIIa control	-	14 days	30/30	100.0%

TABLE V

EFFECT OF PHENOL ON TUMOR CELLS

Group	Phenol 1%	Cell suspension	Saline	Final concn.	Positives / total mice
I	5.0 ml	cells from 1.0 ml	-	1.0%	0/5
II	4.0 ml	1.0 ml	-	0.8%	0/5
III	2.0 ml	2.0 ml	-	0.5%	4/5
IV	1.0 ml	2.0 ml	1.0 ml	0.25%	5/5
V	1.0 ml	2.0 ml	5.0 ml	0.125%	5/5
VI control	-	cells only	-	-	5/5

4-day intervals. Fourteen days after the last vaccination, the mice were challenged with 5,000 tumor cells. The results on the 20th day after the challenge are given in Table VI. The protection resulting from the administration of phenol-killed vaccine was weak.

Experiment 8. Lowest concentration of merthiolate required to kill the tumor cells. Tumor cell suspension with 5×10^5 cells per ml was prepared and mixed with 1:1000 merthiolate solution and saline in varying proportions as shown in Table VII in order to obtain the desired concentration of merthiolate. This suspension was kept at room temperature for one hour and then in the refrigerator for 16 hours. It was then injected intraperitoneally in mice in doses of 0.2 ml per mouse. Washed untreated cells were used as controls. The results in Table VII show that a merthiolate concentration of 1:5000 was the lowest concentration that would kill the tumor cells under the specified conditions. This concentration was employed for killing tumor cells in the preparation of vaccines.

Experiment 9. Immunization of mice with merthiolate-killed tumor cells. Three intraperitoneal injections of 0.2 ml of the merthiolate-killed cells were given at 4 day intervals. Fourteen days after the last immunizing injection, the mice were challenged with 5,000 tumor cells. The results recorded on the 20th day after the challenge are shown in Table VIII. Only 11 of the 30 immunized mice developed the tumor after challenge while 30 of 30 control mice developed the tumor after a similar challenge.

Experiment 10. Minimum dose of ultraviolet light required to kill the ascites tumor cells. Ten ml aliquots of a 1:100 dilution of the

TABLE VI

IMMUNIZATION WITH PHENOL-KILLED TUMOR CELLS

Group	No. of vaccn.	Positives / total mice	% Positives
I	4	29/40	72.5%
II controls	-	39/40	97.5%

TABLE VII

EFFECT OF MERTHIOLATE ON TUMOR CELLS

Group	Merthiolate 1:1000	Cell suspension	Saline	Final concn.	Positives / total mice
I	2.0 ml	2.0 ml	-	1:2000	0/5
II	1.0 ml	2.0 ml	2.0 ml	1:5000	0/5
III	1.0 ml	2.0 ml	7.0 ml	1:10000	1/5
IV	0.5 ml	1.0 ml	6.0 ml	1:15000	5/5
V control	-	cells only	-	-	5/5

TABLE VIII

IMMUNIZATION WITH MERTHIOLATE-KILLED TUMOR CELLS

Group	No. of vaccn.	Positives / total mice	% Positives
I	3	11/30	36.3%
II controls	-	30/30	100.0%

ascitic fluid were exposed in Petri dishes to ultraviolet light for different lengths of time as shown in Table IX. Two-tenths ml of this ultraviolet-treated fluid was injected intraperitoneally into mice within 30 min after irradiation. Unirradiated fluid containing the same concentration of cells was used as a control. An exposure of 10 min or more was required to kill the tumor cells. Ten and fifteen min exposures were employed for the preparation of vaccines.

Experiment 11. Immunization of mice with ultraviolet light-killed tumor cells. Four intraperitoneal injections of 0.2 ml of vaccines irradiated for 10 and 15 min were given at 4-day intervals. Two weeks after the last vaccination the mice were challenged with 5,000 tumor cells. The results obtained on the 20th day after the challenge are given in Table X. Only 5 of 30 mice vaccinated with tumor cells irradiated for 10 min and only 4 of 30 mice vaccinated with the vaccine irradiated for 15 min developed the tumor after challenge. All the 30 unvaccinated controls developed ascites.

III. Non-specific immunization:

Experiment 12. Non-specific immunity induced by incomplete Freund's adjuvant mixture. Forty CFW female mice were injected intraperitoneally with 0.2 ml of incomplete Freund's adjuvant mixture. Another control group consisting of 40 mice received sterile saline instead of the adjuvant mixture. One week later both groups were challenged with 5,000 tumor cells. Observations were recorded on the 20th and 28th days after the challenge. The results shown in Table XI indicate that Freund's adjuvant has a marked capacity to increase the

TABLE IX

EFFECT OF ULTRAVIOLET LIGHT ON TUMOR CELLS

Group	Time of irradn. in min	Positives / total mice
I	8	2/5
II	10	0/5
III	15	0/5
IV	20	0/5
V	25	0/5
VI control	-	5/5

TABLE X

IMMUNIZATION OF MICE WITH ULTRAVIOLET LIGHT IRRADIATED CELLS

Group	Time of irradn.	Positives / total mice	% Positives
I	10 min	5/30	16.5%
II	15 min	4/30	13.2%
III controls	-	30/30	100.0%

TABLE XI

EFFECT OF INCOMPLETE FREUND'S ADJUVANT

Group *	Positives/total no. of mice on 20th day	28th day
I (F. Adjuvant)	3/40	5/40
II (Saline)	34/40	39/40

* Each group consisted of 40 female CFW mice.

resistance of the mice toward an intraperitoneal challenge of the tumor.

Experiment 13. Duration of non-specific resistance induced by incomplete Freund's adjuvant mixture. Mice treated with 0.2 ml incomplete Freund's adjuvant mixture were challenged with 5,000 tumor cells at different intervals as indicated in Table XII. Mice treated with saline instead of Freund's adjuvant served as controls. The results given in Table XII and Fig. 3 show that the protective effect of Freund's adjuvant is not manifested until 5 to 8 days after its administration. On the 10th and 15th day the mice were highly resistant to the tumor. These mice were actually immune to the challenge of 5,000 tumor cells since they were still tumor-free 45 days after the challenge.

Experiment 14. Non-specific immunity induced by zymosan. Five-hundredths gm (0.05 gm) of zymosan was suspended in 10.0 ml saline and to 8.0 ml of this suepnsion was added 2.0 ml of saline. This suspension now contained 400 µgm of zymosan in 0.1 ml. Two-tenths ml of this suspension was injected intraperitoneally into each mouse. The controls received 0.2 ml of sterile saline. The mice were then challenged with 5,000 tumor cells at different intervals of time as shown in Table XIII. The results (Table XIII and Fig. 4) indicate that the non-specific immunizing effect of zymosan was highest when it was given simultaneously with the tumor. This effect decreased gradually until the seventh day when little or no effect was demonstrable.

Experiment 15. Non-specific immunity induced by killed Bordetella pertussis cells. Two billion B. pertussis cells suspended in 0.2 ml of saline were injected intraperitoneally into female CFW mice. The control

TABLE XII

DURATION OF EFFECT OF INCOMPLETE FREUND'S ADJUVANT

Group	No. of mice	Treated with Adj.	Treated with Saline	Time of challenge	No. of positives on 20th day	No. of positives on 28th day
I	10	+	-	-24 hrs	6/10	7/10
II	5	-	+	-24 hrs	5/5	5/5
III	10	+	-	0 hrs	6/10	7/10
IV	5	-	+	0 hrs	5/5	5/5
V	10	+	-	24 hrs	6/10	6/10
VI	5	-	+	24 hrs	5/5	5/5
VII	10	+	-	5 days	6/10	6/10
VIII	5	-	+	5 days	5/5	5/5
IX	10	+	-	10 days	0/10	0/10
X	5	-	+	10 days	5/5	5/5
XI	10	+	-	15 days	1/10	1/10
XII	5	-	+	15 days	5/5	5/5

* These mice were again observed on the 45th day after challenge. In group IX, one mouse had developed ascites while the other 9 did not. In group XI, all the nine mice remained healthy at this time.

FIGURE 3.

NON-SPECIFIC IMMUNIZING EFFECT OF INCOMPLETE FREUND'S ADJUVANT

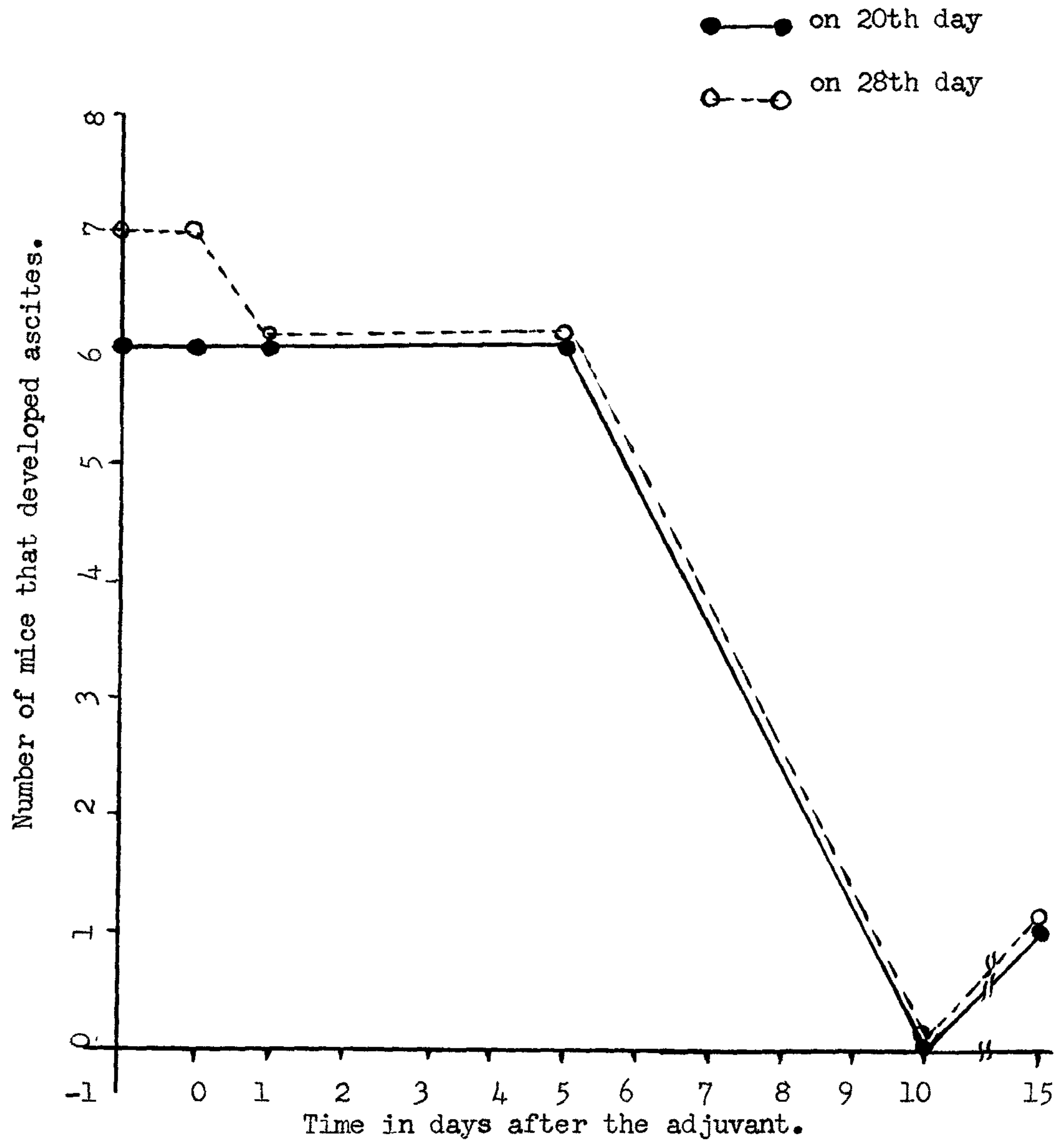


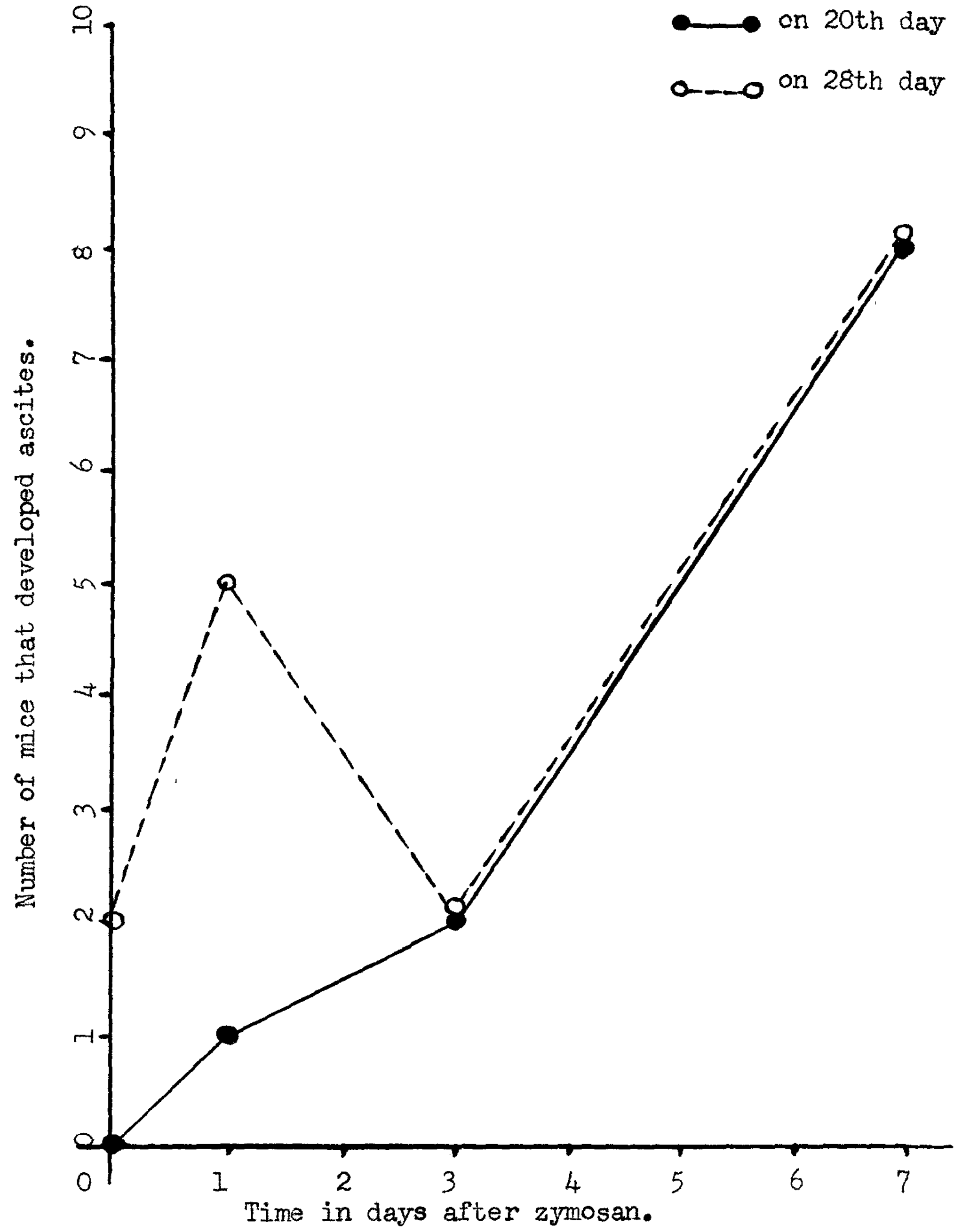
TABLE XIII

NON-SPECIFIC IMMUNITY INDUCED BY ZYMOSAN

Group	No. of mice	Treatment zymosan	Treatment saline	Time of challenge	No. of positives on 20th day	No. of positives on 28th day
I	10	+	-	0 hrs	0/10	2/10
II	10	-	+	0 hrs	10/10	10/10
III	10	+	-	24 hrs	1/10	5/10
IV	10	-	+	24 hrs	10/10	10/10
V	10	+	-	3 days	2/10	2/10
VI	10	-	+	3 days	9/10	10/10
VII	10	+	-	7 days	8/10	8/10
VIII	10	-	+	7 days	10/10	10/10

FIGURE 4.

NON-SPECIFIC IMMUNIZING EFFECT OF ZYMOSAN



mice received 0.2 ml of saline instead of the B. pertussis cells. Subsequently, these mice were challenged with 5,000 tumor cells at different intervals of time as shown in Table XIV. B. pertussis cells, when injected simultaneously with or after the tumor had very little effect in enhancing the defense of the mice towards the tumor challenge (Table XIV and Fig. 5). However, there was a progressive increase in immunity until the 5th day after B. pertussis treatment when 8 of 10 mice did not develop ascites on the 20th day after challenge and 6 of 10 mice did not show any evidence of tumor growth on the 28th day after the challenge. This protective effect of B. pertussis decreased after 5 days, and little protection was observed 18 days after B. pertussis treatment.

Experiment 16. Non-specific immunity induced by Escherichia coli lipopolysaccharide. Two and five-tenths mg of the endotoxin were dissolved in 25.0 ml of saline. This solution contained 100 μgm of endotoxin per ml. Two-tenths ml of this solution containing 20 μgm of the endotoxin was injected intraperitoneally into each mouse. The controls were injected with 0.2 ml sterile saline. These mice were challenged with 5,000 tumor cells at different intervals of time as shown in Table XV. As is evident from the results in Table XV and Fig. 6, there was an increased resistance to tumor transplants when the lipopolysaccharide was given simultaneously with, or 24 hours after the challenge. This protective effect was still noticeable 24 hours after the administration of the endotoxin, but decreased progressively thereafter, until the 10th day when little or no protection could be demonstrated.

TABLE XIV

NON-SPECIFIC IMMUNITY INDUCED BY B. PERTUSSIS CELLS

Group*	Treatment B. pertussis	Treatment Saline	Time of challenge	No. of positives on 20th day	No. of positives on 28th day
I	+	-	-24 hrs	7/10	9/10
II	-	+	-24 hrs	10/10	10/10
III	+	-	0 hrs	5/10	7/10
IV	-	+	0 hrs	10/10	10/10
V	+	-	5 days	2/10	4/10
VI	-	+	5 days	10/10	10/10
VII	+	-	8 days	6/10	6/10
VIII	-	+	8 days	10/10	10/10
IX	+	-	12 days	3/10	5/10
X	-	+	12 days	10/10	10/10
XI	+	-	18 days	6/10	6/10
XII	-	+	18 days	10/10	10/10

* Each group consisted of 10 female CFW mice.

FIGURE 5.

NON-SPECIFIC IMMUNIZING EFFECT OF B. pertussis CELLS

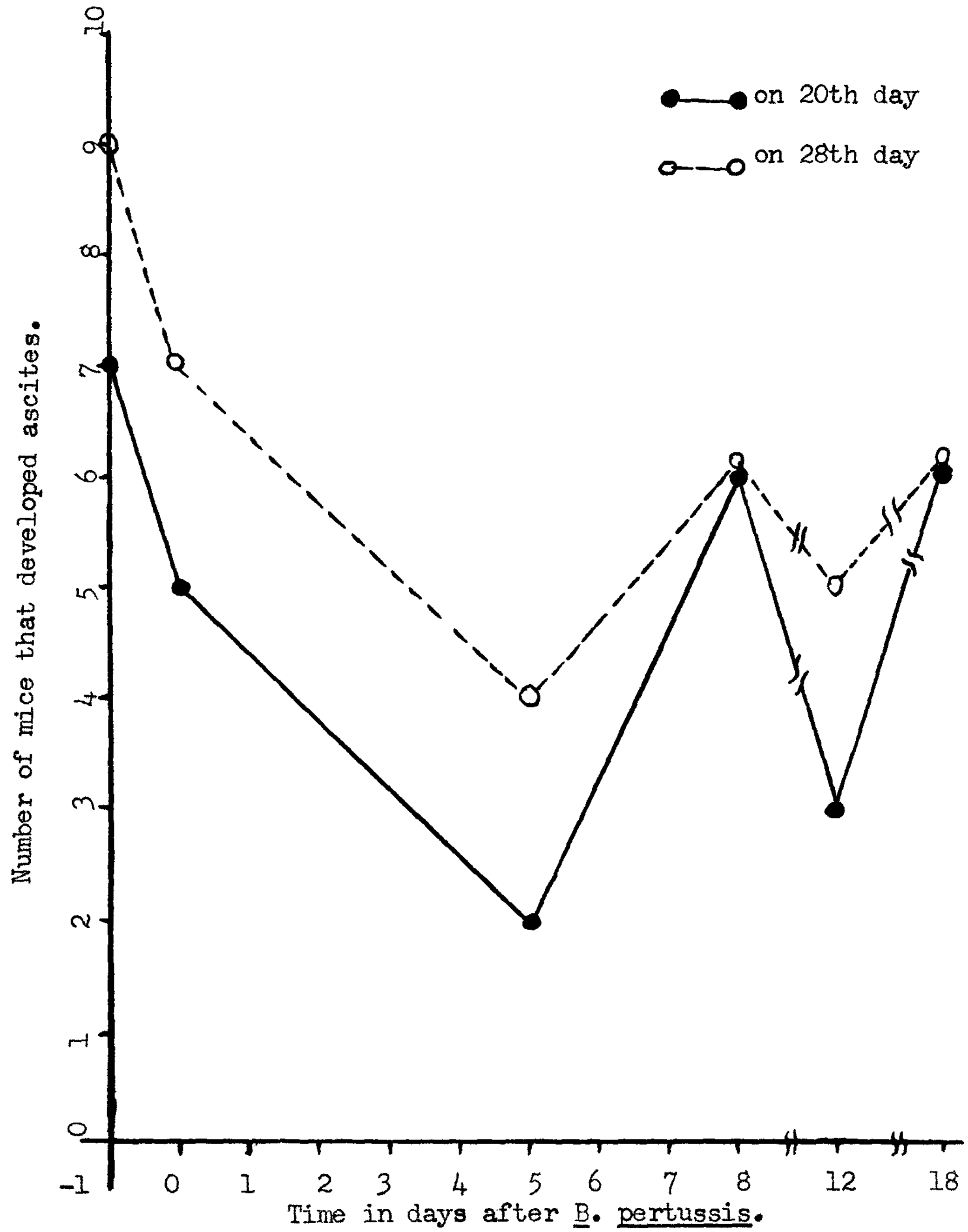


TABLE XV

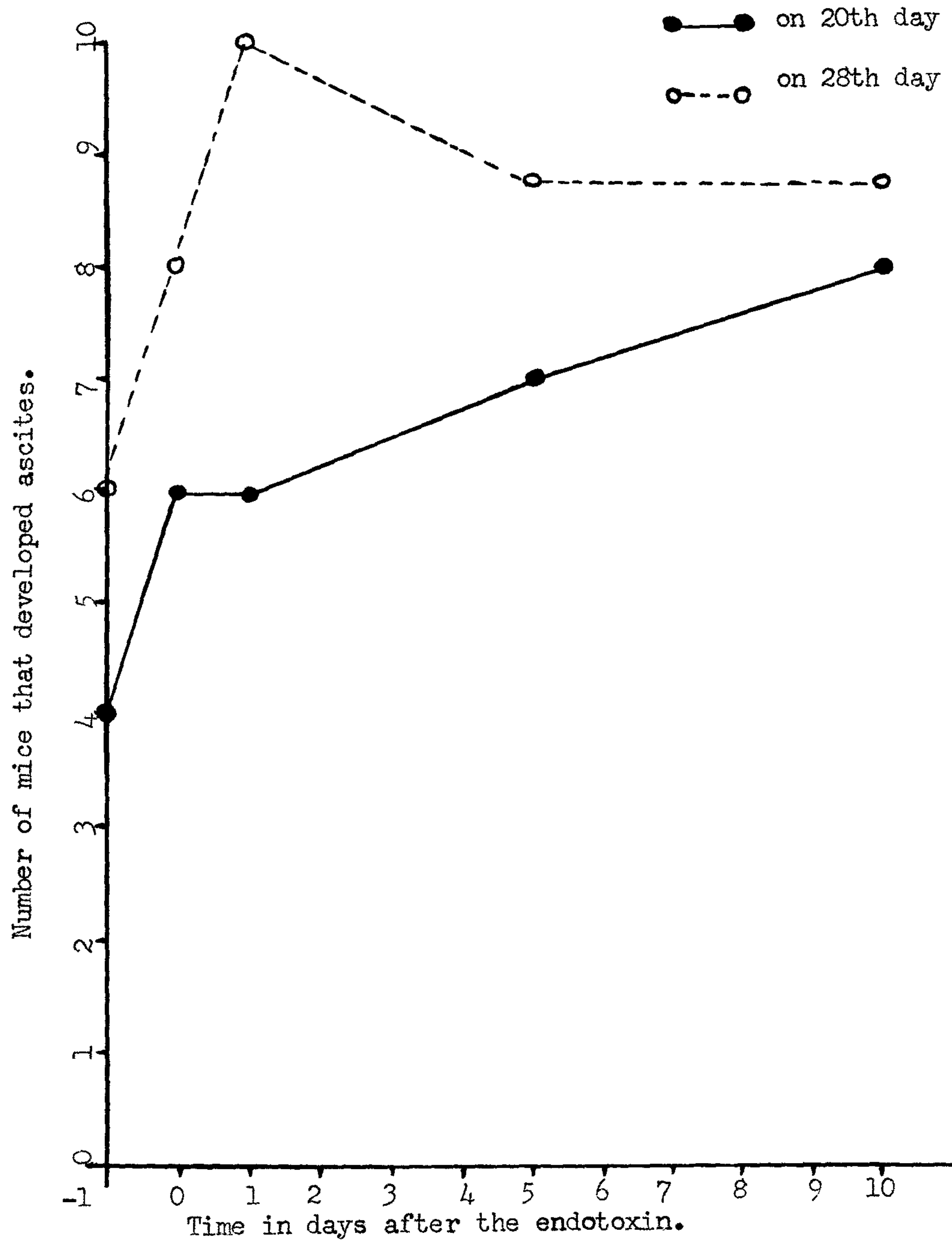
NON-SPECIFIC IMMUNITY INDUCED BY E. COLI ENDOTOXIN

Group*	Treatment endotoxin	saline	Time of challenge	No. of positives on 20th day	28th day
I	+	-	-24 hrs	4/10	6/10
II	-	+	-24 hrs	4/5	5/5
III	+	-	0 hrs	6/10	8/10
IV	-	+	0 hrs	5/5	5/5
V	+	-	24 hrs	6/10	10/10
VI	-	+	24 hrs	5/5	5/5
VII	+	-	5 days	7/10	9/10
VIII	-	+	5 days	5/5	5/5
IX	+	-	10 days	8/10	9/10
X	-	+	10 days	5/5	5/5

* The controls (groups II, IV, VI, VIII and X) consisted of 5 mice in each group, whereas the other groups consisted of 10 mice.

FIGURE 6.

NON-SPECIFIC IMMUNIZING EFFECT OF E. coli ENDOTOXIN



IV. Effect of rabbit antiserum:

Experiment 17. In vitro neutralization of the tumor by rabbit antiserum. A 1:80 dilution of rabbit antiserum (agglutinin titer 1/2560) which produced strong agglutination (4+) was used. Washed and sedimented tumor cells were resuspended in this dilution to obtain a final concentration of 20,000 cells per ml. As a control a similar cell suspension was prepared in normal rabbit serum obtained from the same rabbit before immunizing injections were given. A second control consisted of a saline suspension containing 20,000 tumor cells per ml. These 3 suspensions were incubated in a water bath at 37° C for 1 hour. Half an hour later the tubes were shaken and 0.2 ml of the corresponding suspension injected into each of 10 mice. The results were recorded on the 20th and 28th days after challenge (Table XVI). These results show that the tumor cells are almost completely neutralized after incubation with the antiserum, while not affected by normal rabbit serum or saline.

Experiment 18. Passive immunization with rabbit antiserum. Sixty female CFW mice were divided into 6 groups of 10 mice each. They were all challenged with 4,000 tumor cells, and subsequently given 1 to 5 intraperitoneal injections of 0.1 ml of the undiluted antiserum (titer 1/2560) at different intervals as shown in Table XVII. The results, recorded on 20th and 28th day after the challenge, indicate that treatment with antiserum 24 hours before the challenge had only slight protective effect, but two or more injections given on 0 and 1 day after the challenge, completely protected mice against the tumor transplant. Normal rabbit serum and saline failed to produce this effect.

TABLE XVI

IN VITRO NEUTRALIZATION WITH ANTISERUM

Group*	Cells treated with	Positives / total mice 20th day	Positives / total mice 28th day
I	antiserum	1/10	1/10
II	N.R.S.	10/10	10/10
III	saline	10/10	10/10

* Each group contained 10 female CFW mice.

TABLE XVII

PASSIVE IMMUNIZATION WITH ANTISERUM

		Time of injecting the reagent					No. of positives on	
Group	-24 hrs	0 hr	1 day	2 days	4 days	8 days	20th day	28th day
Antiserum:								
I	+	-	-	-	-	-	1	5/10
II	-	+	-	-	-	-	-	0/10
III	-	+	+	-	-	-	-	2/10
IV	-	+	+	+	-	-	-	0/10
V	-	+	+	+	+	-	-	1/10
VI	-	+	+	+	+	+	-	0/10
Normal Rabbit Serum:								
I	+	-	-	-	-	-	5	5/5
II	-	+	-	-	-	-	5	5/5
III	-	+	+	-	-	-	5	5/5
IV	-	+	+	+	-	-	5	5/5
V	-	+	+	+	+	-	5	5/5
VI	-	+	+	+	+	+	4	5/5
Saline:								
I	+	-	-	-	-	-	5	5/5
II	-	+	-	-	-	-	5	5/5
III	-	+	+	-	-	-	5	5/5
IV	-	+	+	+	-	-	5	5/5
V	-	+	+	+	+	-	5	5/5
VI	-	+	+	+	+	+	5	5/5

Experiment 19. Neutralization of the tumor with antiserum absorbed with washed tumor cells. Rabbit antiserum absorbed with tumor cells (as described in the section on Materials and Methods) was used. A 1:80 dilution of this absorbed antiserum was prepared and in it were suspended washed tumor cells to make a final cell concentration of 20,000 cells per ml. Similar suspensions were prepared in 1:80 dilutions of agglutinating and normal rabbit serum, to be used as controls. These cell suspensions were incubated at 37° C in a water bath for 1 hour and 15 min later were injected intraperitoneally into mice (0.2 ml per mouse). The results obtained on the 20th, 28th and 40th day after challenge are shown in Table XVIII. These results show that antiserum absorbed with tumor cells failed to neutralize the oncogenic property of the tumor cells.

Experiment 20. Passive immunization with heated and specifically absorbed antiserum. Groups of 10 mice were challenged with 5,000 tumor cells and subsequently given 3 intraperitoneal injections of 0.1 ml of the respective antisera at 0, 24 and 72 hours after the tumor transplantation. Agglutinating antiserum heated in a water bath at 56° C for 1 hour was used as heated antiserum. The results shown in Table XIX indicate that no passive immunization was possible with the absorbed antiserum. Heated antiserum, however, retained its immunizing property.

Experiment 21. In vitro neturalization with non-specifically absorbed antiserum. Rabbit antiserum absorbed with normal mouse liver and spleen (as described in the section on Materials and Methods) was used for this experiment as well as experiment No. 22. Normal rabbit

TABLE XVIII

EFFECT OF SPECIFICALLY ABOSRBED ANTISERUM

Group	Cells treated with	Positives/total no. of mice on 20th day	28th day	40th day
I	Antiserum	0/10	0/10	0/10
II	Absorbed antiserum	8/10	9/10	9/10
III	N.R.S.	10/10	10/10	10/10

TABLE XIX

EFFECT OF HEATED AND ABSORBED ANTISERUM

Group	Cells treated with	Positives/total no. of mice on 20th day	28th day	40th day
I	Antiserum	0/10	0/10	0/10
II	Heated antiserum	0/10	0/10	1/10
III	Absorbed antiserum	9/10	10/10	discarded
IV	N.R.S.	10/10	10/10	discarded
V	Saline	10/10	10/10	discarded

serum and specifically absorbed antiserum were used as controls. The dilutions used were 1:10 and 1:120. The results shown in Table XX indicate that non-specific absorption of the antiserum lowered its ability to neutralize the tumor cells, but did not render it completely inactive.

Experiment 22. Passive immunization with non-specifically absorbed antiserum. The antiserum used was the same as for experiment 21, above. Groups of 10 mice were challenged with 5,000 tumor cells and subsequnently treated with the respective serum. Three injections of 0.1 ml of the serum were given 0, 24 and 72 hours after the tumor transplant. Controls were treated with unabsorbed, specifically absorbed and normal rabbit serum. The results shown in Table XXI indicate that non-specific absorption did not affect the protective power of the antiserum when compared to that of the unabsorbed antiserum.

TABLE XX

IN VITRO EFFECT OF NON-SPECIFIC ABSORPTION

Group	Cells treated with		Positives/total no. of mice on 20th day	28th day	40th day
I	1:10	Antiserum	0/5	0/5	1/5
II	1:120	Antiserum	0/5	0/5	1/5
III	1:10	Sp. absorbed antiserum	5/5	5/5	discarded on 28th day
IV	1:120	Sp. absorbed antiserum	5/5	5/5	discarded on 28th day
V	1:10	Non-sp. abs. antiserum	1/5	3/5	3/5
VI	1:120	Non-sp. abs. antiserum	4/5	4/5	4/5
VII	1:10	N.R.S.	5/5	5/5	discarded on 28th day
VIII	1:120	N.R.S.	5/5	5/5	discarded on 28th day

TABLE XXI

IN VIVO EFFECT OF NON-SPECIFIC ABSORPTION

Group	Cells treated with	Positives/total no. of mice on 20th day	28th day	40th day
I	Antiserum	0/10	1/10	5/10
II	Sp. absorb. antiserum	7/10	10/10 discarded	-
III	Non-sp. abs. antiserum	0/10	2/10	5/10
IV	N.R.S.	5/10	10/10 discarded	-

CHAPTER V

DISCUSSION

The experimental results seem to indicate that tumor cells treated with ultraviolet light, formalin and merthiolate (thimerosal) can induce immunity against a subsequent challenge of about 5,000 tumor cells. Ultraviolet light gave the best results (about 80% protection). In demonstrating this immunity one has to bear two facts in mind: 1) actively immunized mice may tolerate only small challenge doses since the induced immunity may not be sufficiently strong to protect the animals against heavier challenges, and 2) the time after immunization, at which the challenge transplant is given is important because the induction of immunity is a gradual process, which reaches its maximum level at different times depending on the type of antigen, the amount given, the route of administration and perhaps other factors. The time factor is illustrated by the results obtained with formalinized vaccines. A challenge given 6 days after the last vaccination was tolerated by 27.5% mice, whereas that given on the 14th day was tolerated by 80%.

In order to explain the possible mechanism of action of agents which render the tumor cells capable of inducing active immunity, one should consider the homograft reaction. Apparently, the tumor grows and kills the host because it fails to evoke the antibody response necessary for its rejection. This may be due to the fact that the genetic make-up of the tumor cells is such as to make them an isologous transplant rather than a heterologous or homologous transplant.

According to the genetic concept of the transplantation reaction introduced by Gorer (45), any agent that is able to alter the genetic make-up of the tumor cells in such a way that it becomes non-allelomorphic to the normal mouse tissue, will render the tumor cells antigenic to the mouse. The previous experiments with x-rays (62) and nitrogen-mustard (63), and our observations with ultraviolet light and formalin, seem to indicate that all these agents that have been employed successfully for immunization experiments, belong to the same group of agents called "mutagenics" (90). If mutations were responsible for this change in antigenicity, one would expect the tumor cells to multiply after they have been treated with the agent. This might be true at least in case of the x-irradiated tumor cells (89).

Another possible mechanism by which immunity could be induced would involve the presence of an active antigen in the tumor cells to which the host may develop antibodies. In normal mice these antibodies will not develop rapidly enough to protect the animal against the rapidly multiplying tumor cells. However, according to this hypothesis, any agent that is able to destroy the carcinogenic property of the tumor cells without affecting the "specific protective antigen," could convert the tumor cells into a vaccine capable of protecting the animal against subsequent transplants. That this hypothesis may indeed be true is indicated by the reports demonstrating specific antibodies in animals with actively growing tumor cells (52, 53), and in rabbits immunized with specific antigens obtained from neoplasms (54, 55). Specific neutralizing antibodies have also been demonstrated in chickens

3 to 5 weeks after the regression of Rous sarcoma (51). The main difficulty here appears to be the fact that in the presence of actively growing tumor cells it is difficult to demonstrate specific antibodies, although they have been demonstrated against Shope Papilloma and Brown-Pearce Carcinoma in rabbits (52, 53).

Still another possible way in which these vaccines could stimulate immunity is through a non-specific host-defense mechanism. Earlier work by Pellimer, Landy and Shear (69), has shown that tissue-polysaccharides derived from normal and malignant (Sarcoma 37 and Carcinoma 241-6) mouse tissue, have the property of increasing the host resistance to bacterial infections by altering the serum properdin levels. Repeated injections of killed tumor cells may cause this effect. The fact that non-specific immunity would ordinarily not be expected to offer complete protection in as great a percentage of mice as specific immunity would, bears but little significance in view of the non-specific immunity that has been induced through treatment with incomplete Freund's adjuvant mixture in our experiments.

The fact that phenol-killed and merthiolate-killed tumor cells produced only weak immunity, can probably be explained by assuming that these agents destroyed or altered the antigenicity of the tumor cells, so that they were not capable of evoking a strong antibody response.

Results with agglutinating antiserum prepared in rabbits indicated that this antiserum was capable of neutralizing *in vitro* the carcinogenic property of the tumor cells. This agrees with the results of Flax (80). This antiserum also protected mice against a challenge of

5,000 tumor cells if 3 or more intraperitoneal injections of 0.1 ml of the antiserum were given. This protective property of the antiserum was lost after repeated absorption with tumor cells, strongly suggesting the presence of specific antibodies to the tumor. Specifically absorbed antiserum also lost its agglutinating capacity, while antiserum absorbed with normal mouse tissues still retained the agglutinins as well as its protective properties against the tumor. Earlier results of Flax (80) and Horn (81) indicated that treatment of mice with antiserum only prolonged their survival period. The challenge used by these workers was large (0.1 ml of the tumor fluid or about 1×10^7 tumor cells per mouse). In our experiments complete protection was obtained with a challenge dose of 5,000 tumor cells. Contrary to the findings of Flax (80), guinea pig complement was not found necessary to show _in vitro_ neutralization of tumor cells. In the present work it has been clearly shown that normal rabbit serum does not have the capacity to neutralize the tumor cells either _in vitro_ or _in vivo_.

The antiserum prepared against washed tumor cells will certainly be expected to contain antibodies against normal mouse tissue, since the mice used were not genetically homogeneous. The role that these antibodies might have played in the present work was ruled out by absorbing the antiserum with normal mouse tissue and showing that its protective property was not removed in this way. The results obtained by Horn (81) indicated that antisera prepared against normal mouse liver and spleen tissue had no neutralizing effect on the Ehrlich ascites tumor cells. This in conjunction with our observation that

absorption of the antiserum with tumor cells removed the agglutinating as well as neutralizing power of the antiserum, favors the postulation that specific antibodies may be responsible for the protective activity of the immune serum.

From the results presented in this thesis, it is also evident that in addition to specific immunization, non-specific agents also are capable of producing immunity to Ehrlich ascites tumor. Bacterial endotoxins and lipopolysaccharides from plant and animal sources have been known to alter the resistance of the host towards infections if given in very small doses (70, 71, 72, 73). In our experiments, zymosan, E. coli endotoxin, B. pertussis cells and incomplete Freund's adjuvant appeared to increase the resistance of mice towards subsequent tumor transplants. Not only the survival time was prolonged, but in the case of Freund's adjuvant, the mice appeared to tolerate completely a challenge of 5,000 tumor cells. The mechanism involved here is not yet known. Various host defense mechanisms have been incriminated. The increase in lysozyme, properdin and phagocytic acitivity of cells have been chiefly considered. As shown by Hook and his co-workers (87), the serum lysozyme and properdin levels showed an initial drop followed by a marked rise after an intraperitoneal injection of 1.0 mg of zymosan in mice. Serum lysozyme did not rise above normal levels until about the 3rd day after the administration of polysaccharide. The properdin level took still longer to show a rise above normal. Ten days after the administration of zymosan, the properdin level was about 160% of normal values, and remained high for about 32 days. The serum

lysozyme level dropped after about 16 days. Earlier work of Pellimer, Landy and Shear (64), had shown that the injection of tissue polysaccharides derived from animal tissues led to a rise in properdin level noticeable 12 to 24 hours after administration of the polysaccharide. The results obtained with zymosan and _E. coli_ endotoxin could possibly be explained on this early rise of properdin level. In the case of Freund's adjuvant and _B. pertussis_, this explanation does not seem to apply. However, according to Hook _et al_. (87), properdin and lysozyme level and the leukocyte count showed marked rise only about 7 days after zymosan injection, and the properdin level remained high for 32 days. If this is the case when Freund's adjuvant is injected, the properdin system might well explain the protective activity of this agent. The protective effect of _B. pertussis_ may be different. Properdin level might not be the only factor involved, however, since Hook _et al_. (87) have shown that after the administration of zymosan, the properdin level reached its peak 10 days after injection of the substance and it remained high for about 30 days. The protection against Ehrlich ascites tumor obtained in the present work did not follow this pattern except in the case of Freund's adjuvant.

It is interesting to note that the protection produced by the agents tested, did not manifest itself at the same time. With zymosan, for example, the protective effect was demonstrated within 24 hours, and disappeared after 6 days. With _B. pertussis_ cells, the maximum protection was noticed on or about the 6th day, while with Freund's adjuvant the protection was marked only 7 days after and persisted for at least

15 days. These facts seem to suggest that more than one non-specific mechanism may be involved for these agents. A study of serum properdin level, lysozyme level and the phagocytic index following the administration of these agents is suggested. This may give a clue to the probable mechanism of action of these non-specific substances.

CHAPTER VI

SUMMARY

Attempts were made to induce active immunity in female Swiss Webster mice against Ehrlich ascites tumor by the use of tumor cells treated with formalin, merthiolate (thimerosal), phenol and ultraviolet irradiation as vaccine. This immunity was tested against an intraperitoneal challenge of 5,000 tumor cells given 2 weeks after the last immunizing injection. It was found that cells treated with ultraviolet irradiation produced maximum protection (86.8%). With formalin- and merthiolate-treated cells, the protection obtained was 80.2% and 63.7% respectively. Only weak protection (27.5%) was obtained with cells treated with phenol.

Non-specific immunizing effect of incomplete Freund's adjuvant mixture, zymosan, killed B. pertussis cells and E. coli endotoxin was also studied. It was found that 9 of 10 mice that received 0.2 ml of the adjuvant mixture intraperitoneally were completely immune to an intraperitoneal challenge of 5,000 tumor cells. This protective effect was noticeable 7 days after the administration of the oil mixture and persisted for at least 15 days. With zymosan, this effect was demonstrated within 24 hours after intraperitoneal injection of 800 μgm of the polysaccharide and disappeared after 6 days. Five days after an intraperitoneal injection of 2 billion merthiolate-killed B. pertussis cells, 8 of 10 mice were found to be more resistant to a transplant of 5,000 tumor cells as compared to the mice that did not receive B. pertussis cells. This effect was found to be decreasing after the 5th

day but was still noticeable 18 days after the injection of B. pertussis cells. E. coli endotoxin, when given intraperitoneally in doses of 20 µgm, was found to increase slightly the resistance of mice. This effect became noticeable with a challenge given 24 hours before the endotoxin and persisted till 10 days after. However, from the results with E. coli endotoxin it appeared that the immunity stimulated by the endotoxin was expressed only by prolonging the appearance of ascites and survival time of the mice. Many of the mice that did not show any ascites on the 20th day were found to develop ascites on the 28th day. Freund's adjuvant on the other hand, appeared to produce complete protection against the challenge used since the mice did not show any ascites even 45 days after the challenge. In mice immunized with zymosan and B. pertussis cells, a comparison of the results on the 20th and 28th day appeared to indicate some degree of complete protection as many of these mice did not show any ascites on the 28th day which was the last day of observation.

It was found that rabbit antiserum to tumor cells was able to neutralize in vitro the carcinogenic property of the Ehrlich ascites tumor cells if they were incubated in an appropriate dilution (1:80) at 37° C for 1 hour. It was also possible to passively immunize mice with 3 or more intraperitoneal injections of 0.1 ml of the antiserum, against a transplant of 5,000 tumor cells. Absorption of this antiserum with tumor cells resulted in loss of both in vitro and in vivo neutralizing properties. Non-specific absorption with normal mouse

liver and spleen resulted in a decrease in the agglutinin titer (from 1:1280 to 1:512) but did not appear to affect its property to neutralize tumor cells either *in vitro* or *in vivo*. It was also found that heating the antiserum at 56° C for 1 hour did not affect its agglutinating or neutralizing property.

The probable mechanisms of action of these specific and non-specific immunizing agents were discussed.

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AUTOBIOGRAPHY

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