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Immunological aspects of the Shope rabbit fibroma virus

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IMMUNOLOGICAL ASPECTS OF THE
SHOPE RABBIT FIBROMA VIRUS

by

M. BENJAMIN PERRYMAN
B. S., Emory University, 1970

presented in partial fulfillment of the requirements
for the degree of
Master of Science
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Approved by:

Chairman, Board of Examiners

Dean, Graduate School

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Chapter I

INTRODUCTION

The Shope rabbit fibroma was discovered in 1932 by Richard E. Shope of the Rockefeller Institute in Princeton, New Jersey, when he observed small fibromatous tumors under the skin of a wild cottontail rabbit which he shot. The tumor was transmissible by means of tissue filtrates to wild cottontail and domestic rabbits. The tissue filtrates given to domestic rabbits produced fibromatous tumors similar to those found naturally (usually on the feet) in wild cottontail rabbits. When transmitted by inoculation to either the domestic or the cottontail rabbit, the tumors grew initially quite rapidly but became stationary and regressed in about 9-12 days, leaving the rabbit immune to subsequent infection (Allison, 1966).

The Natural Reservoirs of Shope Rabbit Fibroma

The natural reservoirs of Shope rabbit fibroma appear to be the wild cottontail rabbit. Kilham and Woke (1953) demonstrated that fleas and Aedes aegypti mosquitoes fed on fibromatous tumors on wild cottontail rabbits transmitted typical fibromas, usually on the feet and legs, to healthy cottontail rabbits. These insects retained the ability to transmit the tumor for at least 2 weeks after feeding on a tumor. In 1955 Kilham and Dalmat found that three other species of mosquitoes, Anopheles quadrmaculatus,
Culex pipens, and Aedes triseriatus, were as effective in transmitting the Shope rabbit fibroma virus as Aedes aegypti. They also found that the infected mosquitoes carried the virus, which was localized in the head parts, for as long as several weeks. The Shope rabbit fibroma, though it can be easily transmitted by culture filtrates and insect bites, is not contagious.

**Relationship of Fibroma and Myxoma Virus**

Fibroma and myxoma viruses appear to be very closely related, although the diseases they produce are quite different. Fibroma virus, normally not fatal, produces a local, benign, fibromatous tumor which usually regresses in domestic and cottontail rabbits, and is not transmitted by direct contact. Myxoma virus induces an acute, generalized disease transmissible by direct contact. The disease is fatal only for domestic rabbits, whereas cottontail rabbits develop only a transient localized infection evidenced by a fibromatous tumor at the site of inoculation. This lesion is similar to that induced by fibroma virus in cottontail rabbits. The similarity in these lesions led Shope (1932) to postulate an immunological relationship between the two viruses. He found that recovery from an infection with fibroma virus decreased susceptibility to a subsequent infection with myxoma virus.

After recovery from infection with fibroma virus, domestic rabbits inoculated with myxoma virus developed a
local myxoma which showed little tendency to generalize. Most of the animals recovered, suggesting that fibroma infection provided some degree of protection against myxoma virus. Cross-immunity was very difficult to demonstrate in the opposite direction as almost all domestic animals with primary myxomatosis died. The very rare rabbit surviving primary myxomatosis proved to be immune to subsequent inoculations with either myxoma or fibroma virus.

From these studies it is evident that animals infected with fibroma or myxoma virus develop cross-immunity and that the viruses are closely related (Shope, 1936). The closeness of this relationship was demonstrated in 1939 by Berry and Dedrick, who accomplished the transformation of fibroma virus into myxoma virus. Myxoma virus was heat-inactivated at either 60°C or 75°C and mixed with live fibroma virus. This preparation, when injected into the testicles of normal domestic rabbits, frequently produced generalized myxomatosis leading to death of the animal. A control of heated myxoma alone did not produce disease. These findings were later confirmed by Hust (1937), Shope (1950) and Smith (1952).

Kilham (1957 and 1958) found that the fibroma—myxoma transformation could be accomplished in tissue culture by adding heat-inactivated myxoma virus (67°C for 40 minutes) to live fibroma virus growing in either rabbit kidney cells
or rabbit testis cells. The mechanisms of this transformation are not known, but an exchange of genetic information must be supposed.

**Physical and Chemical Characteristics**

Myxoma and fibroma viruses have many characteristics in common, neither agglutinate red blood cells (Fenner, 1953), and both are sensitive to ethyl ether and produce acidophilic intracytoplasmic inclusion bodies in the epithelial cells of infected skin lesions and in the ectodermal cells of the chorioallantoic membrane of chicken embryos. The diameter of the virus particles is approximately 175 nm (Schlesinger and Andrews, 1937) by filtration and centrifugation techniques, while electron microscopy gives a diameter of about 230-280 nm. The viruses are closely related to vaccinia, fowlpox and ectromelia (Fenner, 1953; Gaylord and Melnick, 1953) except that the latter three are not susceptible to ethyl ether. Cross-immunization and gel diffusion tests indicate that rabbit fibroma virus is more closely related to myxoma than to squirrel fibroma virus (Woodroffe and Fenner, 1965). Electron micrographs have shown that fibroma and myxoma viruses have typical pox virus morphology (Farrant and Fenner, 1958; Gaylord and Melnick, 1953; Morgan, *et al.*, 1954; Dernhard, 1958) and are similar in structure (Barnhard, *et al.*, 1954; Lloyd and Kahler, 1955; Scherrer, 1968). Barnhard, *et al.* (1954)
examined ultra-thin sections of fibroma lesions and found the virus to be an ovoid particle, approximately 230 nm in diameter. Outside of the infected cell the virus may have a brick-shaped structure (Scherre, 1968). The virus particles are usually found in the cytoplasm of the infected cell, often aggregate near the cell nucleus, and are visible as Feulgen-positive inclusion bodies by light microscopy (Constantin and Febvre, 1956 and 1958).

**Tissue Cultures**

Faulkner and Andrews (1935) and later Kilham (1956) propagated fibroma virus in tissue culture of rabbit testis. This cultured virus was fully infective to rabbits. The titer of virus increased after each passage and a maximum titer of $10^{-4}$ was reached in about two weeks after which a gradual decline began concomitantly with a decrease in viability of the tissue culture cells. No cell death was attributable to virus proliferation. Constantin and Bauer (1956) reported similar results.

Growth of the virus in tissue culture cells was accompanied by the formation of acidophilic intracytoplasmic inclusion bodies (Kilham, 1956; Constantin and Febvre, 1956, 1958; Constantin, et al. 1956) similar to those noted by Shope (1932) in fibromas from wild cottontail rabbits.

The virus caused cell destruction in primary kidney and skin cultures from newborn rabbits (Verna and Eylar,
1962) and in cell monolayers, produced plaques measuring from 1 to 2 mm in diameter within four to five days. Immune serum accentuated the production of these plaques (Moore and Walker, 1962). The virus titers determined by the plaque technique were similar to those obtained by the titration in the skins of rabbits. Hinze and Walker (1964) successfully propagated fibroma virus, not only in primary kidney cell cultures, but also in serially-cultured rabbit kidney cells. After infection these cells lost contact inhibition and numerous intracytoplasmic inclusion bodies were observed. The transformed cells were able to induce formation of tumors when inoculated into the cheek pouch of the hamster.

**Induction of Generalized Fibromatosis in Rabbits**

Although fibroma virus usually produces a mild, benign and localized lesion in rabbits, under some circumstances generalized and fatal fibromatosis can be produced. Andrews and Ahlstrom (1938) observed that the regression of viral lesions normally seen was modified by an intramuscular injection of tar. Some of the subcutaneous tumors grew progressively and produced metastases. Generalized fibromatosis, sometimes fatal, resulted after intravenous (i.v.) injection of virus into tarred animals. A single dose of the tar, or of benzpyrine and other carcinogens, on the day of virus inoculation was sufficient to produce these
results. A fibrosarcoma, isolated from a rabbit which had received 2 intramuscular injections of tar 4 months apart followed by an i.v. injection of fibroma virus, was transplanted through a series of 12 rabbit passages. The first of these transplanted tumors regressed, but many of the tumors of later passages grew progressively and some metastases occurred. Virus particles could not be isolated from the tumor. By giving 300-700 R of total body irradiation 24 hours prior to i.v. injection of fibroma virus, generalized fibromatosis could also be induced (Clemmesen, 1939). Treating rabbits with cortisone after inoculation with fibroma virus also retards the regression of the tumors and sometimes results in generalized fibromatosis (Harel and Constantin, 1954; Allison and Friedman, 1966). Treatment of infected animals with methotrexate retards tumor regression and may lead to metastases (Allison and Friedman, 1966).

**Fibromatosis in Newborn Rabbits**

Inoculation of fibroma virus into newborn rabbits produces either a generalized, fatal disease or a generalized or local nonfatal fibrosarcoma. Occasionally, a metastasizing sarcoma can be induced. The age of the rabbit and the dose of inoculum are the deciding factors for the type of disease produced (Duran-Reynals, 1940, 1945; Allison, 1966).
Immunological Aspects

Duran-Reynals (1940, 1945) found that after injection of virus into adult domestic rabbits, virus was present in the viscera during the first 8 days of infection, but viremia did not develop. Immunity to reinfection was demonstrable 24 hours after infection and the antibody response was prompt and vigorous. However, following similar injections into newborn animals, virus could be detected in the blood for at least 13 days and in the viscera for at least 21 days. Immunity to reinfection was not well established 12 days after infection, the antibody response developed slowly, and the titers were low. When large amounts of virus were injected into newborn rabbits, an acute lethal disease with destructive, inflammatory, and proliferative features resulted. This disease closely resembled fatal myxomatosis of adult domestic rabbits. If small amounts of virus were injected into newborn rabbits, a progressively-growing tumor and metastases resulted, occasionally followed by fatal generalized fibromas. In some cases, the tumor regressed after reaching a large size. These generalized lesions were similar to the generalized fibromas obtained in rabbits prepared by carcinogens, immunosuppressants, or whole body irradiation followed by intravenous injection of fibroma virus.

Allison (1966) and Allison and Friedman (1966), in a series of experiments with adult and newborn domestic
rabbits, concluded that recovery from initial infections and immunity to subsequent infections with rabbit fibroma virus was due to a cell-mediated immune response. Adult animals inoculated with Shope rabbit fibroma developed tumors which began to regress on about the 9th day. Delayed-type hypersensitivity reactions could be demonstrated by intradermal injection with virus antigen on the 5th day, and circulating antibody could be demonstrated on the 7th day. Rabbits inoculated with virus shortly after birth developed tumors which grew progressively. Circulating antibody could be demonstrated in the newborns on the 7th or 8th day, but no delayed-type reaction could be elicited even by the passive transfer of lymph node cells into the skin of adult rabbits bearing fibromas.

In adult domestic rabbits treated with methotrexate, an inhibitor of delayed-type hypersensitivity and antibody formation, regression of fibroma was retarded to the extent that the tumors became larger than those in control rabbits. Secondary fibromas developed at sites distant from the original inoculation site. Secondary injections of fibroma virus into rabbits infected with fibroma virus and treated with methotrexate on the 5th and 7th days induced fibromas at these sites, while untreated, infected animals were resistant to reinfection. Hyperimmune antifibroma serum administered to the methotrexate-treated rabbits prevented the formation of secondary fibromas but did not produce
necrosis or regression of the fibromas. In animals treated with cortisone, delayed hypersensitivity was suppressed, but some antibody was formed. Regression of fibromas in these animals was retarded, and no secondary lesions developed. From these results it was postulated that regression of the fibromas is not due to interferon or any other factor inherent in the fibroma cells but to a cell-mediated immune response. Humoral antibody was thought to prevent the formation of secondary fibromas but not to be important in the regression of the primary tumor.

Tompkins, et al. (1969) developed an in vitro measure of cellular immunity to the fibroma virus; a modification of the macrophage inhibition test employing infected monolayers of PRK cells as the source of antigen. The capacity of these monolayers of infected cell homogenates to inhibit migration of peritoneal exudate cells from fibroma-infected rabbits paralleled the onset of a cutaneous delayed hypersensitivity response and resistance to reinfection in the intact animal. Studies using antigens prepared at different intervals after infection of cells with the virus suggested that the test is a measure of specific antigen(s) associated with the cell surface rather than those components of the intact viron. Immune serum did not prevent the antigen from inhibiting migration of sensitized cells but enhanced inhibition.
Tompkins, et al. (1971) characterized the surface antigen on cells infected by fibroma virus which developed a surface antigen detectable by immunofluorescence. Sera from rabbits with regressed fibroma-induced tumors reacted with the surface of viable fibroma-infected cells, but not with cells infected with vaccinia virus, herpesvirus. Serum prepared against vaccinia virus, herpesvirus SV40, and rabbit papilloma virus did not react with fibroma-infected cells. The surface antigen seems to develop late and to depend upon DNA synthesis. The antibody did not react with intact virions attached to the cell surface. The antigen persisted in association with the cell membrane long after infectious virus had decreased to low levels. A temporal relationship between the surface antigen measured by the immunofluorescent technique and the antigen measured by the macrophage inhibition test could be demonstrated. Antibody reactive against the surface antigen developed in rabbits with growing fibromas, and the antibody titer increased as the tumor regressed.

**Statement of Problem**

Based upon previous work by Allison (1966) and Allison and Friedman (1966) this study was proposed to further elucidate the immune mechanisms involved in recovery from Shope rabbit fibroma. Humoral and cell-mediated aspects of the problem were investigated as well as specific and
non-specific components of the immune response. Adult and neonatal rabbits were employed.
Chapter II

MATERIALS AND METHODS

Animals

Adult New Zealand rabbits of both sexes, weighing 5-7 kg, obtained from local breeders were used throughout the investigation. Newborn rabbits of mixed breed from a breeding colony maintained by the author were also employed. All newborn animals were injected with virus at 36 hours of age and weaned at 6 weeks. The adult animals were given food and water ad libitum.

Virus Strain

The Patuxent strain of the Shope rabbit fibroma was obtained from the American Type Culture Collection (ATCC Lot No. 2). The strain, of an unknown passage in rabbits, was donated by Dr. Lawrence Kilham. The virus was propagated in rabbit testes which were harvested, ground, and resuspended in Minimum Essential Medium (MEM), Grand Island Biological Supply Co., Oakland, California. The cultured virus was placed in 1 ml volumes in sealed ampules and stored at -70°C. This stock virus induced tumors when .2 ml of a 10^-6 dilution was given to rabbits. All animals were injected intradermally with .2 ml of a 10^-4 dilution of the original virus suspension.
**BCG Strain**

The Paris strain of *Mycobacterium bovis*, Bacille-Calmette-Guerin (BCG) maintained on Dubos medium, Difco, Detroit, Michigan, was employed for vaccination of the animals. A .2 ml volume of a suspension containing \(2.4 \times 10^7\) organisms per ml, adjusted to the concentration by use of a Klett-Summerson Photoelectric colorimeter, New York, was used. The organisms were injected intravenously (i.v.).

**Skin-Test Antigens**

Two-tenths ml of PPD (NIH, Bethesda, Maryland) containing 8 ug was used for skin tests. To test for skin sensitivity to the virus .2 ml of a \(10^{-1}\) and \(10^{-2}\) dilution of the stock virus suspension heated at 56°C for 30 minutes was used.

**Preparation of Immune Serum, Buffy Coat, Peritoneal Exudate Cells (PEC) and Spleen Cells**

The flanks of rabbits were shaven and 10 intradermal (i.d.) injections of virus suspension were given. When tumor began to regress, the animals received 30 ml of sterile mineral oil intraperitoneally (i.p.). Seventy-two hours later they were bled by cardiac puncture and sacrificed. Blood from half of the animals was drawn in versine-coated syringes (to prevent clotting) and 10 ml amounts were administered i.v. to experimental animals. Blood of the
remaining animals was allowed to clot, centrifuged, and the serum was removed. The serum was also given i.v. in 10 ml doses. The clots were gently teased through a #60 stainless steel mesh into saline, centrifuged, and the peripheral white blood cells (buffy coat) were removed. Cells were counted in a hemocytometer and adjusted to a concentration of $1 \times 10^7$ cells per ml. Experimental animals received i.v. 2 ml of this suspension. To collect the peritoneal cells, sterile MEM was injected into the peritoneal cavity and then withdrawn together with the oil and placed in a cold separatory funnel. The aqueous layer containing the cells was withdrawn and the cells were washed twice in MEM, counted in a hemocytometer and adjusted to a final concentration of $1 \times 10^7$ cells per ml. Two ml of this suspension were given i.v. into each experimental animal. The spleens from immune rabbits were teased through a stainless steel screen, washed 7 times in MEM, the cells were counted in a hemocytometer and adjusted to a concentration of $1 \times 10^7$ cells per ml. Two ml of the spleen suspension were given i.v. to each experimental animal.

Measurement of Tumors and Delayed Reactions

Two diameters of the lesions were measured at right angles to one another and the approximate area was calculated by multiplying the two values. Thickness of the lesion was measured using a "Schnelltester" system kroplin.
Volumes of tumors were calculated by multiplying area by thickness. Delayed skin reactions were measured in the same manner and the volumes were calculated by using the formula suggested by Waksman, 1958:

\[ V = (W \times L \times \frac{1}{2}T)^{0.75} \]

measured at right angles to one another

- \( V \) = volume of lesion in \( \text{mm}^3 \)
- \( W \) = width of lesion in \( \text{mm} \)
- \( L \) = length of lesion in \( \text{mm} \)
- \( T \) = thickness of lesion in \( \text{mm} \)

**Anti-Lymphocyte Serum (ALS)**

Horse anti-(rabbit lymphocyte) serum (Microbiological Associates Lot NO. 10972) was used as an immunological depressant in newborn animals. This serum was used either unabsorbed or absorbed with normal rabbit red blood cells.

**Virus Neutralization Test**

Two-fold dilutions of the immune serum to be tested were made in sterile phosphate-buffered saline. To each of the dilutions of serum (0.2 ml), 0.2 ml of virus suspension was added to give a final dilution of \( 10^{-4} \) of virus. The mixtures were incubated for 30 minutes at 37\(^\circ\)C, and then injected (0.2 ml) into the skin of normal rabbits. The development of lesions was observed for 10 days. The neutralization titer of the serum was taken as the highest dilution inhibiting tumor development.
Isolation of Immunoglobulin

The method used was that of Stelos as detailed in Weir's Handbook of Experimental Immunology (1967). A 125 ml volume of saturated ammonium sulphate solution (SAS), adjusted to pH 6.5 with concentration ammonium hydroxide, was added to 350 ml of serum (this gives a 26.3% saturation of SAS). The mixture was allowed to stand 15 minutes and then centrifuged. The sediment was washed twice with 100 ml of 40% SAS, and resuspended in .9% NaCl, reprecipitated twice with 50% SAS, and washed as above. The final sediment was then resuspended in .9% NaCl, dialysed against .9% NaCl, and finally dialysed against distilled water. The precipitated protein was centrifuged and resuspended in phosphate-buffered saline pH 7.2 (PBS). This globulin solution was divided into two equal portions and one portion was treated with 0.6% 2-mercaptoethanol (2-ME), Sigma, St. Louis, Missouri. The 2-ME-treated portion was dialysed against PBS. A 5 ml aliquot was lyophilized and weighed to determine the amount of globulin. Animals received 9.4 mg of globulin in 5 ml.

Preparation of Soluble Mediators Derived from Rabbit Lymphocytes Presensitized with Complete Freund's Adjuvant (CFA)

The basic procedure was that of Mooney and Waksman (1970). The popliteal and axial lymph nodes were harvested from animals previously sensitized with CFA. The lymph
nodes teased through sterile stainless steel mesh into #199 media (Grand Island Biological Supply Co., Oakland, California) with 100 units of penicillin, 100 ug streptomycin per ml. The cells were washed once in 100/Earle's (Grand Island Biological Supply Co., Oakland, California) and centrifuged at 250 x g for 10 minutes. Cells were resuspended in #199 and counted in a hemocytometer. This cell suspension was then passed through a separatory funnel packed with 60 mesh glass beads. The glass bead column had previously been incubated at 37°C for ½ hour, covered with #199 media containing 20% normal rabbit serum which had been heated at 56°C for 30 minutes and centrifuged at 27,000 x g. After placing the cells on the column, it was incubated at 37°C for 30 minutes. The cells and media were then drawn off the column and the column was washed with #199 media with 20% normal rabbit serum until the elute was no longer cloudy. The suspension was centrifuged at 250 x g for 10 minutes to collect the cells. The cells were counted and adjusted to a concentration of 1 x 10^7 cell per ml in #199 media containing 5% normal rabbit serum, 100 units of penicillin and 100 ug streptomycin per ml. The lymphocyte suspension prepared in this manner was 90% pure. Six ml quantities of the final cell suspension were placed in 30 ml Falcon Plastic tissue culture bottles (Falcon Plastics, Los Angeles, California). They were incubated for 24 hours at 37°C. One-half of the bottles contained purified
protein derivative (PPD) at a concentration of 25 ug per ml and the other half without PPD. After the incubation period the supernatants were pooled and centrifuged at 400 x g for 10 minutes. The resulting supernatants were stored at -70°C until needed.
Chapter III

RESULTS

Immunity in Adult Rabbits

Passive Transfer of Immunity

The initial studies were performed to determine if resistance to SRF could be transferred from animals recovered from fibromatosis to normal animals. Allison (1966) and Allison and Friedman (1966) reported that a cell-mediated immune response is responsible for recovery and immunity to SRF infections. Spleen cells, PEC, whole blood, and serum from immune rabbits were injected i.v. into groups of 4 animals each, 72 hours after i.d. inoculation of 0.2 ml of MEM containing a $10^{-4}$ dilution of virus. Animals in group 1 received $2 \times 10^7$ PEC, those in group 2 were given $2 \times 10^7$ spleen cells, those in group 3 were injected with 10 ml of whole blood, and those in group 4 received 10 ml of serum. Animals in group 5 were not treated. The surface area of the tumor was determined daily. The results of this experiment are shown in Figure 1.

Spleen cells and PEC were eliminated from the protocol of later experiments, as they seemed to offer no protection. It was also decided to separate whole blood into serum and peripheral white blood cells. Four groups of four animals were used; animals in group 1 received 10 ml of immune

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Figure 1. The surface area of fibromas produced in rabbits infected by intradermal injection of a $10^{-4}$ dilution of fibroma peritoneal exudate cells, spleen cells, whole blood or serum from rabbits recovered from fibromatosis.
Figure 2 - The average surface area of fibromas produced in rabbits by intra-dermal injection of a $10^{-4}$ dilution of fibroma virus 72 hours prior to treatment with serum, buffy coat cells and buffycoat cells + serum from animals recovered from fibromatosis.
Figure 3 - The average tumor volume of fibromas produced in rabbits by intradermal injection of a $10^4$ dilution of fibroma virus 72 hours prior to treatment with serum, buffy coat cells, and buffy coat cells + serum from animals recovered from fibromatosis.
serum; those in group 2 were given $2 \times 10^7$ buffy coat cells, and those in group 3 were injected with 10 ml of immune serum plus $2 \times 10^7$ buffy coat cells (i.v.) 72 hours after infection. A control group (4) received no treatment. The areas of the tumors were calculated daily (Figure 2). The results from these two studies indicate that resistance to the SRFV cannot be transferred with immune spleen cells or PEC. Some resistance can be transferred using immune serum and immune serum plus buffy coat cells. The second experiment was repeated using groups of seven animals each. The volumes of the lesions rather than their areas were determined in this experiment (Figure 3). No significant resistance was transferred in this experiment.

**Soluble Mediators**

In some instances soluble products of specifically-stimulated lymphocytes (soluble mediators) have been shown to suppress the growth of tumors (Bernstein, et al., 1971). Soluble mediators were prepared and harvested as previously described. The supernatants were tested to determine whether they possessed the ability to induce a reaction similar to delayed cutaneous hypersensitivity. A 0.2 ml volume of supernatant fluid from PEC of rabbits sensitized with CFA which had been incubated in the presence of PPD was injected i.d. into two separate sites on the flanks of each of 2 normal rabbits. The same amount of supernatant
fluid from the same sensitized cells incubated without PDD was injected into 2 other sites on these animals. Measurements of the reactions were taken at 6, 12, 24 and 48 hours (Table 1). The soluble mediators possessing the ability to induce a skin reaction were tested for their ability to suppress the growth of fibromas. Stock fibroma virus was diluted $10^{-4}$ in the soluble mediator. Two-tenths ml of this mixture was injected i.d. into 5 sites on each side of 4 rabbits. Two control rabbits were inoculated with an equal amount of virus without soluble mediator. Skin reactions due to SRF were of the same magnitude as those previously noted. The tumors in the experimental animals enlarged and regressed at the same rate as those on the control animals. Thus, despite a strong skin reaction induced with soluble mediator, no protection was provided against tumor induction by the virus.

**Resistance to Reinfection**

Allison (1966) inoculated rabbits with fibroma virus at two i.d. sites and, at daily intervals thereafter, the animals were injected on a different site with infectious virus. Tumors developed at the original site as well as at sites inoculated with virus on days 2 to 4. At the site infected with virus on day 5 a delayed-type response occurred, but no tumor developed. A similar procedure was employed in this investigation to test susceptibility to reinfection with the virus. Five rabbits were injected
i.d. with 0.2 ml of MEM containing a $10^{-3}$ dilution of the stock virus suspension, and all animals developed tumors which were first palpable on day 4. On this day and for 5 succeeding days, the animals received a single i.d. injection of the same amount of virus. No tumors developed on any of the animals, with the exception of the tumor at the original inoculation site. Contrary to Allison's results, injection of live virus failed to provoke delayed responses. Heat-killed suspensions of $10^{-1}$ and $10^{-2}$ dilutions of the original suspension injected i.d. on day 10 also failed to produce delayed reactions.

**BCG Therapy in Normal Animals**

Live BCG has been used in several instances to retard the growth of tumors and leukemias (Zbar, et al., 1971; Schwartz, et al., 1971; Mathe, et al., 1969; Larson, et al., 1971). In a study devised to determine the effect of injection of viable BCG into established fibromas, 7 normal animals were injected i.d. at 6 sites on each flank with $10^{-4}$ dilution of the original virus solution. As soon as the tumors were palpable (4 days) .2 ml of a BCG suspension containing approximately $4 \times 10^6$ organisms was injected into the base of the tumors present at alternate sites. During the first few days the tumors at the sites treated with BCG continued to grow at the same rate as those at the untreated sites. Later measurement of the size of the tumors on the
Table 1. Volume of skin reactions induced in normal rabbits by intradermal injection of 0.2 ml of fluid harvested after incubation of peritoneal exudate cells of rabbits sensitized with CFA with or without PPD.

<table>
<thead>
<tr>
<th>Time of Reading</th>
<th>Ave. Vol. of 4 Sites Injected with Super. from Cells Incubated with PPD</th>
<th>Ave. Vol. of 4 Sites Injected with Super. from Cells Incubated w/o PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>28.6 mm$^3$</td>
<td>0</td>
</tr>
<tr>
<td>12 hours</td>
<td>34.2 mm$^3$</td>
<td>0</td>
</tr>
<tr>
<td>24 hours</td>
<td>35.2 mm$^3$</td>
<td>0</td>
</tr>
<tr>
<td>48 hours</td>
<td>9.2 mm$^3$</td>
<td>0</td>
</tr>
</tbody>
</table>

\( a = \) Volumes calculated by formula suggested by Waksman, 1958.

Formula \((W \times L \times T^{0.75}) = V\)

- \(V\) = volume of lesion in mm$^3$
- \(W\) = width of lesion in mm measured at right angles to one another
- \(L\) = length of lesion in mm
- \(T\) = thickness of lesion in mm

\( b = \) Two sites on each of two rabbits
treated animals became difficult since the reaction to BCG produced an added volume to the tumors. More metastases were observed in the treated animals than in the controls.

**BCG Therapy in BCG-Immune Animals**

A similar study was performed using BCG-immunized animals. Five rabbits were given 0.2 ml of BCG suspension i.v. containing $2.4 \times 10^7$ viable units per ml. Three weeks later the animals were skin-tested by i.d. injection of 0.8 ug of PPD in 0.2 ml volume, and all gave strongly positive skin-test reactions. Three non-immunized animals were employed as controls. All of the animals were injected i.d. at 6 sites on each flank with a $10^{-4}$ dilution of the stock virus suspension. When the tumors were palpable (4 days) they were injected into the base with either $4 \times 10^6$ viable BCG organisms, 8 ug of PPD, or normal sterile saline in volumes of 0.2 ml. Alternate tumors were treated with one of the preparations. Delayed skin reactions occurred at the site of injection of BCG and PPD in the immunized animals and reactions to BCG occurred in the non-immunized animals. All tumors, however, enlarged at approximately the same rate regardless of treatment.

**Non-Specific Immunity with BCG**

Another study was performed to determine whether BCG-immunized animals possessed non-specific immunity toward fibroma infection. Ten animals were injected i.v. with
.2 ml of a BCG suspension containing $2.4 \times 10^7$ viable units per ml. Three weeks later the animals were skin-tested with 8 ug PPD in .2 ml saline and all gave strong skin reactions. The BCG-immunized animals and 5 normal animals were injected i.d. with .2 ml of a $10^{-4}$ and $10^{-2}$ dilution of heat-inactivated original stock virus suspension in .2 ml. Five BCG-immune rabbits served as control. The skin-test volumes are presented in Table 2.

**Immunity in Newborn Animals**

**Resistance Induced by Passive Transfer of Immune Serum**

Tumors induced by fibroma virus in newborn rabbits have been reported to grow progressively until the host dies (Duran-Reynals, 1945; Allison, 1966). The initial investigation of this problem was an attempt to transfer immunity from adult animals which had recovered from a fibroma infection to infected newborn animals. Adult animals were bled by cardiac puncture 3 weeks after infection with fibroma virus, and were skin-tested with $10^{-1}$ and $10^{-2}$ dilutions of the original suspension of heat-inactivated virus. All skin tests were negative. The sera were pooled and used in all investigations with immune adult serum. Experimental animals were given a single i.d. injection of a $10^{-4}$ dilution of the original virus suspension 36 hours after birth. The litters of each were then divided into two groups of approximately equal numbers. One group of animals was
injected i.p. with 5 ml of immune serum 24 and 48 hours after infection with virus, and the rabbits in the other group were not treated. The animals were observed for the appearance of tumors (Table 3). At various intervals after the first virus injection, the animals were challenged by i.d. injection of the same amount of virus (Table 4) to demonstrate the presence or absence of active resistance to reinfection with SRF. A total of 10 ml of serum was found to protect almost all animals from virus infection. All of the treated animals developed fibromas with a second virus challenge, while untreated animals did not.

**Titration of Immune Serum**

An attempt was made to determine the amount of immune adult serum needed to protect newborn rabbits which had been previously infected with fibroma virus. All dilutions of serum were given i.p., and equal volumes were given 24 and 48 hours after injection of virus. Rabbits were given an i.d. injection of .2 ml of a $10^{-4}$ dilution of stock virus suspension 36 hours after birth. Five ml of serum was sufficient to protect animals from developing tumors. These animals were observed for the appearance of tumors, and were rechallenged with a single i.d. injection of .2 ml of $10^{-4}$ dilution of the original virus suspension at 8 weeks of age (Table 5).
Figure 4 - Average tumor volume of fibromas of normal rabbits compared to that of rabbits immunized three weeks prior to virus challenge with 0.2 ml of a BCG suspension containing $2.4 \times 10^7$ organisms per ml.
Table 2. Volumes of skin reactions of BCG- and fibroma-immune, fibroma-immune, and BCG-immune animals challenged with $10^{-1}$ and $10^{-2}$ dilutions of heat-inactivated fibroma virus and 8 μg of PPD.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Volume of 8 μg PPD Skin-Test Lesion</th>
<th>Volume of $10^{-1}$ Dilution of Heat-Inactivated Skin-Test Lesion</th>
<th>Volume of $10^{-2}$ Dilution of Heat-Inactivated Skin-Test Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG- and Fibroma-Immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>147.0</td>
<td>16.2</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>30.0</td>
<td>25.6</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>75.6</td>
<td>19.2</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>49.5</td>
<td>7.2</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>159.6</td>
<td>8.4</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>133.9</td>
<td>75.6</td>
<td>6.0</td>
</tr>
<tr>
<td>7</td>
<td>37.1</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>8</td>
<td>99.0</td>
<td>7.2</td>
<td>4.0</td>
</tr>
<tr>
<td>9</td>
<td>19.8</td>
<td>32.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10</td>
<td>24.3</td>
<td>6.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Average</td>
<td>77.6</td>
<td>20.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Fibroma-Immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>32.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>7.2</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Average</td>
<td>0.0</td>
<td>7.8</td>
<td>0.0</td>
</tr>
<tr>
<td>BCG-Immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>130.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>49.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>94.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>92.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Average</td>
<td>89.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Volumes calculated in Table 1.
Table 3. Number of newborn rabbits (36 hours old) developing fibromas following i.d. challenge with a $10^{-4}$ dilution of stock SRFV. Controls not treated with specific immune serum. Treated animals injected i.p. with specific immune serum$^a$.

<table>
<thead>
<tr>
<th>Litter No.</th>
<th>Treated Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./Group</td>
<td>No./Tumors</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ = A total of 10 ml of serum was administered to each treated animal, 5 ml at 24 hours and 5 ml at 48 hours after virus challenge.
Table 4. Number of newborn rabbits developing fibromas on second i.d. challenge with a $10^{-4}$ dilution of SRFV. Controls not treated with specific immune serum. Treated animals injected i.p. with specific immune serum at 50 and 75 hours of age.

<table>
<thead>
<tr>
<th>Litter No.</th>
<th>Age at Second Virus Challenge</th>
<th>Treated Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No./Group</td>
<td>No./Tumors</td>
</tr>
<tr>
<td>1</td>
<td>4 weeks</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>8 weeks</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>6 weeks</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5 weeks</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>7 weeks</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

All control animals developed tumors following primary challenge with same dilution of virus.
Table 5. Effect of varying the amount of specific immune serum given by i.p. injection to newborn animals on the number of animals which develop fibromas.

<table>
<thead>
<tr>
<th>Total Serum Adminis.</th>
<th>No. In Group</th>
<th>No. of Tumors</th>
<th>No. of Tumors When Challenged at 8 Wks.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml.</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2.5 ml.</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>1.25 ml.</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Serum administered in 2 i.p. injections, \( \frac{1}{2} \) the total amount of serum given at 24 hours and the second half at 48 hours after virus challenge.

Animals were infected with a .2 ml injection of a \( 10^{-4} \) dilution of the stock virus suspension at 36 hours of age.
**Serum Fractionation**

Fourteen 36-hour old rabbits were given i.d. .2 ml of a $10^{-4}$ dilution of the stock virus suspension. Five of these rabbits received 9.4 mg of the 2-ME treated globulin preparation, five were given 9.4 mg of the untreated preparation, and four were not treated. The animals were observed for tumor development. None of the treated animals developed tumors.

**Resistance Induced by Transfer of Normal Adult Macrophages**

A group of seven rabbits, 36-hours old, was selected. Each animal was injected i.d. with .2 ml of a $10^{-4}$ dilution of the original virus suspension. Twenty-four hours later 4 animals were given i.p. $6.1 \times 10^7$ normal adult macrophages in 1 ml. All of these animals and the 3 control animals developed tumors in 5 days. The tumors on animals treated with macrophage were larger and metastasis were more prevalent. All of the tumors in both groups regressed at the same rate and eventually disappeared. None of the animals developed tumors when injected i.d. with 0.2 ml of a $10^{-4}$ dilution of the original virus suspension administered when the animals were 5 weeks of age.

**Heat-Inactivated Serum**

Immune adult rabbit serum was heated at 56°C for 2 hours. Two 5 ml i.p. injections were given to 4 animals which had received an injection of a $10^{-4}$ dilution of the
original virus suspension. One serum injection was given 24 hours and the other 48 hours after virus injection. Only one of the four treated animals developed a tumor in 5 days, while all five of the control animals developed tumors in 5 days.

Death Rate in Newborn Rabbits Infected with SRF

Contrary to results obtained by Duran-Reynals (1945) and Allison (1966) no fatalities attributed to the SRFV occurred among the young rabbits used in these studies. Some tumors in newborn animals eventually attained a size approximating 1/3 to 1/2 that of the animal, but all of these eventually regressed. None of the animals recovered from fibromatosis could be reinfected at a later date.

Virus Neutralization Test

A group of nine rabbits was given a single i.d. dose of virus 36 hours after birth, and three of these were treated with 10 ml of specific immune serum. No tumors developed in these animals. The remaining six animals, which were not treated, developed tumors. Eight weeks later serum was obtained from all nine rabbits and a virus neutralization test was performed. There were no neutralizing antibodies in the serum of treated animals; however, the serums of the infected control rabbits contained antibodies with titers ranging from 1:2 to 1:8 (Table 6). When challenged with a single .2 ml injection of a 10^-4 dilution of virus, 24 hours
Table 6. Correlation of virus neutralizing titers against SRFV to the number of fibromas developed on challenge with an i.p. injection of a $10^{-4}$ dilution of the original virus suspension.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>Virus-Neutralizing Titer</th>
<th>Tumor Development Challenged at 36 hr.</th>
<th>Challenged at 8 wks.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Untreated</td>
<td>1</td>
<td>1:2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1:4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1:2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1:8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1:4</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

All animals injected with 0.2 ml of $10^{-4}$ dilution of SRFV i.d. at 36 hours of age. Treated animals given specific immune serum and did not develop tumors on initial challenge. Untreated animals all developed tumors on initial virus challenge. Normal rabbit serum possessed no virus-neutralizing titer. Virus incubated with saline was fully active in tumor induction.
after the serum sample was observed, treated animals developed tumors but untreated rabbits did not. A control of normal rabbit serum had no virus-neutralizing capacity, and a second control of virus incubated in saline was fully active in tumor induction. The immune serum pool had a titer of 1:4.

**Treatment with ALS**

Seven 36-hour old rabbits were injected i.d. with .2 ml of a $10^{-4}$ dilution of the original virus suspension. Four animals were treated with three 1 ml injections of ALS i.p. at 48 hour intervals. Three untreated animals served as controls. The animals were observed for tumor development and growth. The ALS-treated animals developed tumors sooner and the tumors on these animals grew larger than those on untreated animals.
Chapter IV

DISCUSSION

Immunity in Adult Rabbits

Passive Transfer of Immunity

Data presented in Figures 1 and 2 indicate that intravenous injection of serum, whole blood, and peripheral white blood cells plus serum from animals recovered from fibroma infections influences the course of infections with fibroma virus in adult rabbits. In these experiments, injection of the above substances was withheld until 72 hours after the animals were infected in order to allow the virus to become firmly established prior to institution of therapy. Immune serum does contain virus-neutralizing antibodies but these may be ineffective from a therapeutic standpoint. The criterion for protection was reduction of the average area of tumors in the treated animals as compared to that in control rabbits. Spleen cells, peritoneal exudate cells and peripheral white blood cells which were washed to decrease the effect of serum, did not influence the effects of infection.

Evidence obtained by Allison (1966) and Allison and Friedman (1966) indicated that recovery from and immunity to SRF is dependent upon cell-mediated immune responses. If only cell-mediated responses are responsible for immunity as
Allison and Friedman postulate, passive transfer of resistance should be accomplished by transfer of sensitized lymphocytes or macrophages, but not by transfer of serum. Our findings show that passive transfer of cells from immune animals to animals infected 72 hours previously does not confer resistance to infection; however, humoral antibodies do confer some degree of immunity. It appears (Figures 1 and 2) that specific immune serum plus peripheral white blood cells gives the greatest amount of protection to infected recipient animals. A plausible explanation for this observation is a synergistic action of antibodies and cells. The serum could contain opsonizing antibodies which prepare virus particles or induced tumor cells for phagocytosis and subsequent destruction by macrophages in the buffy coat cells. It is possible that greater protection might be afforded if immune serum or immune serum and buffy coat cells were administered soon after infection with virus rather than after 72 hours. Evidence for this will be discussed later. The results in Figure 3, using decreased tumor volume as a criterion, indicate that no significant protection of adult animals was obtained when they were treated with specific immune serum, immune buffy coat cells, or cells with immune serum 72 hours after virus infection. Further study in this area is indicated, especially an investigation of the relationship of time of treatment to the amount of protection given by specific
immune serum.

The above results show that neither spleen cells, PEC, nor circulating cells from rabbits recovered from fibromatosis are capable of conferring resistance to SRFV in adult rabbits infected 72 hours previously with this agent. The influence of immune serum also seems debatable. However, since it is stated that cell-mediated responses are important in the recovery of animals from virus infections, other studies were done to determine if non-specific resistance could be established and if in vitro correlates of cell-mediated immunity could be demonstrated.

Soluble Mediators

Skin reactive factor (SM-SRF) is one of the soluble mediators produced when sensitized lymphocytes are exposed to specific antigen in vitro (Mooney and Waksman, 1970). Following injection into normal animals, SRF produces a lesion which may be considered as a compressed delayed reaction. Specific antigen given i.d. into sensitized guinea pigs produces a reaction which appears in 6 to 10 hours, reaches a peak in 24 to 48 hours, and gradually decreases in size. The same antigen injected i.d. into a normal guinea pig will not produce a reaction unless the agent is a toxic one. On the other hand, SM-SRF injected into a normal animal produces a reaction which develops in 2 to 4 hours, reaches a peak in 6 to 12 hours, and then
gradually disappears. The skin reaction is fore-shortened because the production of the active material has been accomplished in vitro rather than in vivo. Heise, et al. (1960) have shown that production of SRF is not demonstrable in tissue culture until 9 hours after sensitized lymphocytes and specific antigen are mixed and incubated at 37°C. SM-SRF produced in this laboratory by incubation of PEC from rabbits sensitized to CFA together with PPD causes a skin reaction when injected i.d. into normal rabbits. The lesion, however, is well-developed in 6 hours, reaches a peak in 12 to 24 hours, and disappears by 48 hours. Active delayed reactions in sensitized rabbits induced by injection of specific antigen reach their maximum size in 24 to 48 hours and persist for several days. In experiments performed by Bernstein, et al., (1971) the soluble products of specifically-stimulated lymphocytes (soluble mediators) were mixed with ascites cells from the sixth generation of a transplantable hepatoma and injected i.d. into the skin of normal guinea pigs. Tumor growth was suppressed in guinea pigs inoculated with this mixture. The soluble mediator preparation used by Bernstein, et al. possessed the ability to produce a reaction similar in appearance to delayed cutaneous hypersensitivity, as did the preparation used in this study. The SM-SRF reaction, when examined histologically, shows an accumulation of mononuclear cells (Bernstein, et al., 1971). When rabbits were injected with SRF suspended
in soluble mediator prepared from lymphocytes of rabbits specifically-stimulated with CFA, no inhibition of tumor growth was observed. It must be assumed that mononuclear cells which accumulate at the reaction sites are incapable of phagocytosing and destroying the virus particles. This is again evidence against the participation of cell-mediated immunity in immunity against SRF.

**Resistance to Reinfection**

In the original study by Allison (1966) it was found that adult rabbits are immune to reinfection with SRFV days after primary infection. Our experiments confirm the observation that immunity to reinfection is developed by 5 days after infection. Contrary to Allison's results, however, no cutaneous delayed hypersensitivity response could be elicited in infected animals by i.d. injection of either live or heat-inactivated virus.

**BCG Therapy in Normal Animals**

In some instances, (Zbar, et al., 1971) viable BCG injected directly into tumors of normal animals will cause the tumor to regress. In the SRF system, however, increased rate of tumor regression in treated animals was not observed. Viable BCG injected into the base of the tumor causes an active infection at that site. It appears that the lymphoid cells which aggregate at the site of injection of BCG are ineffective against SRF even though a typical tubercle (a
manifestation of delayed hypersensitivity) is formed at the site.

**BCG Therapy in BCG-Immune Animals**

Although strongly positive skin reactions develop when viable BCG or 8 ug of PPD is injected into the base of fibromas present on rabbits previously immunized with BCG, no evidence of protection is observed. From this data, it would appear that the macrophages which assemble in the delayed reaction are incapable of phagocytosing and destroying either the virus particles or the fibroma cells.

**Non-Specific Immunity with BCG**

The results presented in Figure 4 indicate that BCG-immune animals do not possess non-specific immunity toward SRF. Tumors induced in BCG-immune animals appear, enlarge, and regress at the same rate as those in normal animals. Thus, while BCG-induced non-specific immunity does enhance immunity to certain tumors and leukemias in man and animals (Zbar, et al., 1971; Schwartz, et al., 1971; Mathe, et al., 1971; Larson, et al., 1971), it does not appear to be effective in the SRF system. An interesting observation was made when animals in this experiment were skin-tested with PPD and a suspension of heat-inactivated virus. The results in Table 2 indicate that BCG has an adjuvant effect with respect to the induction of specific delayed
hypersensitivity in rabbits subsequently infected with SRF. Although BCG immunization gave no protection, it did increase the volume of skin-test lesions in animals immune to both BCG and SRF. These findings indicate that a delayed skin response to SRF cannot be correlated with immunity to the virus. Delayed hypersensitivity reactions in animals immunized with BCG are much larger than those in animals immunized with SRF, while tumor size, growth, and regression are essentially identical in the two groups. The positive skin-test reactions against SRF observed in animals immunized with BCG and SRF were the only ones obtained in these studies, although SRF-immune animals were skin-tested in an identical manner. It is apparent from discussion presented thus far that no supporting evidence for the participation of delayed hypersensitivity in immunity to SRF was obtained in this study.

**Immunity in Newborn Rabbits**

**Resistance Induced by Passive Transfer of Immune Serum**

Newborn rabbits have been shown to be extremely sensitive to tumor induction by SRFV (Duran-Reynals, 1940, 1945; Allison, 1966; Allison and Friedman, 1966). For this reason passive transfer of resistance by either cells or serum would require that the factor be quite effective. Since the results previously discussed show that lymphoid cells of animals recovered from fibromatosis are not
effective in transferring resistance to normal rabbits, the following studies were undertaken to determine if transfer of immune serum could confer resistance to newborn animals. The results indicate that i.p. injection of 10 ml of specific immune serum into newborn rabbits infected with fibroma virus 24 hours prior to treatment results in almost complete suppression of tumor growth (Table 3). All of the untreated rabbits develop tumors one week after virus challenge. Thus, administration of immune serum to newborn rabbits protects them against infection with SRFV. This protection cannot be attributed to neutralization of the initial inoculum of virus by serum, since the animals were infected 24 hours before treatment. It is interesting to observe that following challenge with a second injection of virus, all of the animals which developed tumors on initial challenge were resistant to reinfection. All animals treated with serum which did not develop tumors on primary challenge did so following the second challenge with virus. The lone animal treated with serum which developed a fibroma following primary challenge with virus was immune to the secondary challenge. These findings indicate that treatment with specific immune serum resulting in prevention of tumor-formation interferes with the animal's ability to develop active immunity to the virus or the tumor cells. The only reason which seems to explain this situation is that immune serum prevents the spread of virus from cell to cell and, as a
consequence, the amount of viral antigen released is insufficient to stimulate the immune system to produce the factors necessary for active immunity. This situation is remindful of that occurring during the treatment of measles with immune serum. Treatment of exposed children too soon after their exposure to a case of measles will prevent infection, but will not result in the production of active immunity. It is evident that passive immunity may be achieved by transfer of specific immune serum to newborn rabbits. The minimal amount of specific immune serum effective in preventing tumor formation by newborn rabbits is 5 ml of the serum pool used in these studies. This represents a large fraction of the total serum pool of these animals. Studies performed using γ globulin preparations indicate that the protecting antibodies are in the γG immunoglobulin.

Heat-Inactivated Serum

Ten ml of serum heated at 56°C for 2 hours given in two i.p. injections to a newborn rabbit infected 24 hours previously with SRFV was still active in the preparation of tumor development. Based upon this evidence, it can be assumed that the active portion of the serum is non-heat labile.

Two in vitro studies support the idea that humoral antibodies are involved in regression of SRF-induced tumors.
Tompkins, et al., (1970) have shown that sera from rabbits with regressed fibromas will react in a specific manner with the surface of viable fibroma-infected cells. This evidence implies that animals infected with fibroma virus produce antibody with the capacity to bind with the surface of infected cells, but gives no indication as to the sequelae of this primary interaction. Singh, et al., (1972) presented evidence that animals bearing SRF-induced tumors produce antibodies cytotoxic to tissue culture cells infected with the virus. This study also showed that 19S antibodies appeared on the 7th day of infection and reached peak titer on day 13, and disappeared by day 17. On the other hand, 7S antibody was barely detectable on day 7, reached maximum titer on day 13, and remained high for at least 50 days.

Resistence Induced by Transfer of Normal Adult Macrophages

The findings of Zisman, et al. (1970) and of Duran-Reynals (1940, 1945), Allison (1966), and Allison and Friedman (1966) demonstrated that young animals are more susceptible to infection with certain viruses than are older animals. It was shown by Zisman, et al. (1970) that administration of macrophages from older mice increased the resistance of suckling mice to infection with herpes virus. The other workers (Duran-Reynals 1940, 1945; Allison 1966; Allison and Friedman 1966) showed that young rabbits infected
with SRFV developed larger tumors than did adult animals. Our studies however, fail to show that administration of macrophages of normal adult rabbits to newborn rabbits induces resistance to infection with SRFV. Rather, it appears that newborn rabbits treated with macrophages of normal adult rabbits develop a greater number of metastatic lesions than do normal suckling rabbits. The metastatic lesions in the treated animal may occur when macrophages engulf virus particles which they are unable to destroy. The virus is then disseminated by the macrophages and freed by their death. A metastatic lesion may then develop if the virus is not phagocytosed and destroyed by another macrophage. This observation would seem to indicate that the animals possess no active immunity to the SRF at this age. If such immunity were present and antibodies active against the virus were produced the observed metastases would not occur. Further study is indicated in this area using specific immune macrophages and normal macrophages given with an amount of specific immune serum insufficient in itself to protect the animal from fibroma infection.

**Virus Neutralization Test**

Data in Table 5 indicates that the virus neutralization titer of a rabbit's serum can be correlated to the animal's resistance to reinfection with the SRF. Animals treated
with specific immune serum which prevented tumor induction on primary challenge developed no virus neutralizing titer and were susceptible to tumor induction when rechallenged at a later date. This evidence supports the concept that true passive immunity is transferred with specific immune serum.

Treatment with ALS

Animals treated with ALS 24 hours after virus infection develop tumors sooner than non-treated control animals. The tumors in the treated animals also grow to a larger size than those in the controls. This is to be expected as ALS depresses the animal's immune system, allowing the tumors to grow to a much larger size.

There are basically two types of specific immunity, humoral and cell-mediated. The former is characterized by specific antibodies and the latter by specifically-stimulated lymphocytes. Humoral immunity may be transferred from one animal to another with serum, cell-mediated immunity may be transferred with lymphoid cells. Both types of immunity may be involved in recovery from viral infections. It appears that in measles infection antibody plays the more important role, while in herpes simplex infection it is cell-mediated immunity (Allison, 1966) which is more important. These are only two examples of many virus infections in
which one type of immunity or the other appears to play
the major role.

In our studies with adult rabbits infected with the
SRFV, it was shown that resistance to the infection could
not be transferred with lymphoid cells from immune animals.
BCG, which enhances the cell-mediated response, was also
ineffective in enhancing resistance to SRFV in both normal
and BCG-immune animals. These results indicate that cell-
mediated immunity plays little if any role in resistance
to SRFV. Animals immunized with both BCG and SRFV did,
however, display a positive delayed cutaneous hypersensi-
tivity reaction when injected i.d. with heat-inactivated
virus. SRFV-immune animals did not give positive skin
reactions when injected in the same manner. This indicates
that a positive delayed cutaneous hypersensitivity reaction
cannot be correlated to cell-mediated immunity against the
SRF. Attempts to transfer resistance to the SRF with immune
serum were not successful in all cases. A possibility exists
that immune serum would be more effective if administered
sooner after virus infection, i.e. 24 hours.

In newborn animals it was observed that immune serum
given 24 hours after virus infection will passively transfer
resistance in virtually all of the treated animals. These
animals, however, did not develop active resistance to the
virus and developed tumors when challenged a second time
with the virus. All control animals which received no
treatment developed tumors on primary virus challenge, but were immune to a second challenge of virus. The activity against SRFV appears to be the IgG portion of the serum. In vitro support for this concept is found in work by Singh, et al. (1972). ALS, which depresses the immune response, lessens an animal's ability to resist fibroma infection. Since ALS inhibits cell-mediated immunity more than humoral immunity, it appears that cell-mediated immunity may also play a role in SRFV resistance. Normal adult macrophages do not inhibit SRFV infection when given to infected newborn rabbits. This again indicates that cell-mediated immunity is less important than humoral antibody in resistance to the SRFV infections.
Chapter V

SUMMARY

The Shope rabbit fibroma virus (SRFV) induces a local, benign tumor which spontaneously regresses in adult rabbits. The virus in some instances induces a fatal generalized disease in newborn rabbits. Previous work by Allison (1966) and Allison and Friedman (1966) attributes resistance to SRFV to a cell-mediated immune response.

Attempts were made passively to transfer immunity from SRFV-immune animals to adult domestic rabbits infected 72 hours previously with the virus. Immune peritoneal exudate cells, immune spleen cells, and immune peripheral white blood cells were ineffective in transferring resistance. Immune serum, immune whole blood and immune serum mixed with immune peripheral white blood cells were effective to a small degree.

Attempts to produce non-specific immunity with BCG and soluble mediator-skin reaction factor were unsuccessful. Delayed hypersensitivity reactions to heat-inactivated virus suspensions appeared only in animals which were immune to both BCG and SRFV.

Passive transfer of resistance by immune serum was accomplished in newborn rabbits infected with virus at 36 hours of age. Five ml of immune serum was sufficient to protect the animals. The active factor in the serum is
non-heat labile and appears to be 7S or IgG immunoglobulin. Animals treated with immune serum after virus challenge at 36 hours developed no tumors, however, when challenged again with virus these animals developed tumors. Animals not treated with serum all developed tumors on initial virus challenge, but were immune to a second virus challenge. Treated animals developed no virus neutralizing titers after initial virus challenge, while untreated animals did.

Newborn animals treated with normal adult macrophages after virus infection developed tumors with more tendency to metastasis than untreated controls.

Treatment with anti-lymphocyte serum caused tumors to appear earlier and grow larger in newborn animals.

From these results we postulate that resistance to SRFV is not completely dependent upon a cell-mediated immune response, but rather humoral antibodies.
List of References


1936. Further observations on the transformation of the virus of rabbit fibroma (Shope) into that of infectious myxomatosis (Sanarelli). (Abstract) J. Bact. 32: 356.


