Experimental Studies on the Etiology of Hodgkin's Disease*

WARREN L. BOSTICK AND LAVELLE HANNA

(Department of Pathology, University of California School of Medicine, San Francisco, Calif.)

For many decades Hodgkin's disease has been looked upon as a type of malignancy that possesses many characteristics which seem to set it apart from most of the other cancerous processes. Many of these characteristics have been ones that suggest an inflammatory background to the tumor, such as the microscopic granulomatous appearance, the fever, the cyclic exacerbations and remissions of the disease, and the partial anergic state. In fact, most of the early workers interested in Hodgkin's disease considered it to be in some way related to known infections.

Excellent and complete recent reviews of Hodgkin's disease are available (Hoster et al. [11], Jackson and Parker [12], and Wallhauser [20]) that present the evidence for and against the virus and nonvirus concepts of the etiology of Hodgkin's disease. A presentation of that same data would serve no purpose here.

During the past decade the technic of using fertile chicken eggs for the study of virus diseases has come into its own. Although used in the study of neoplasms as early as 1913 by Murphy (14) and re-used for experiments with viruses by Goodpasture et al. (9) in 1932, the thorough study of methods by many workers was required before reproducible observations could be made.

This new medium of study, which has proved itself so adaptable to virus research, presented a new approach to the relationship of Hodgkin's disease to any possible virus-like agent. Bostick (3) commenced a preliminary survey of Hodgkin's disease by the systematic use of the various methods of chicken embryo passage. By recording the effect of Seitz-filtered serially passed Hodgkin's disease extract in chicken eggs, he noted a slight but statistically greater mortality of embryos inoculated with Hodgkin's disease material than with carefully prepared control tissue extracts.

Amniotic fluid, harvested after at least from 4 to 15 serial passages, was employed in all observations reported here.

With this provocative initial success, all the possible variations in virus and chicken egg experiments were methodically surveyed. The technics studied are listed below under "Methods." The majority of the methods failed to give any evidence of a difference between the Hodgkin's disease and the control material. However, since all methods were explored at considerable expense of time, animals, and material, it is of value to indicate in this survey all the avenues of investigation used and the results obtained. It is apparent that certain methods should be abandoned as apparently fruitless avenues of research, and others are being pursued with increasing promise.

METHODS AND RESULTS

The first studies were directed toward demonstrating a lethal factor by its direct effect on the embryo or its membranes. Many of the known viruses which can be successfully cultivated in embryonated eggs cause the death of the embryos in a fairly uniform interval of time. Lesions which sometimes develop on the chorioallantois have provided a crude method of determining the amount of certain viruses, and in some viruses specialized tissues of the embryo may show pathologic changes characteristic of the infectious process.

The results of the mortality studies have been reported in detail elsewhere (Bostick [3]). Fresh Hodgkin's disease lymph nodes were ground under sterile conditions, diluted 1:10 in 0.85 per cent NaCl, Seitz-filtered, and inoculated in 0.02-ml. amounts into the amniotic sacs of fertile chicken eggs which had been incubated for 7 days at 38.5° C. Control material consisting mainly of carcinomatous lymph node extracts was treated in an identical manner. Deaths occurring during the 24-hour period immediately following inoculation were considered nonspecific. Mortalities were checked for the next 10-day period. Amniotic fluid was harvested from dead embryos and stored at 4° C. for further transfer. Bacteriologic studies were made at all stages, and upon any secondary contamination the material was Seitz-filtered before subsequent passage. Average mortalities were calculated for each of two groups, based on a total of over 2,700 eggs. In the Hodgkin's disease group A the mortality was 18 per cent higher than in the...
control, and in the Hodgkin's disease group B the mortality was 11 per cent greater than in the controls. Both of those differences were shown to be statistically significant ($P = 0.001$ and $P = 0.005$, respectively). The greatest difference in mortality was shown to occur between the fifth and sixth days.

No specific gross or microscopic lesions could be demonstrated in live or dead embryos. Hematoxylin and eosin stains were used for the general histologic studies, Machiavelli stains for inclusion body studies. Tissues examined included the chorionallantoic and amniotic membranes, lung, and liver from normal control and experimental embryos.

If the effect of any agent under investigation were more debilitating than lethal to the embryo, a difference in weight between control and Hodgkin's disease series might be demonstrable.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>COMPARISON OF EMBRYO WEIGHT IN HODGKIN'S DISEASE AND CONTROL INJECTED SERIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin's Disease</td>
<td>Control</td>
</tr>
<tr>
<td>Dead at 6 days</td>
<td>11</td>
</tr>
<tr>
<td>Alive at 6 days</td>
<td>56</td>
</tr>
<tr>
<td>Alive at 9 days</td>
<td>55</td>
</tr>
</tbody>
</table>

Weights were determined at 6 or 9 days following inoculation (i.e., 15- or 16-day embryos). Dead embryos were weighed as soon as possible after death, live embryos after 2 hours of chilling at 4°C. The difference was so slight as to be insignificant (Table 1).

The protein content of the inoculated eggs was studied and compared. The amount of protein in the amniotic fluid from 12-day live control and experimental eggs was determined. Determinations were based on readings in a Klett-Summerson colorimeter 10 minutes after addition of sulfosalicylic acid. Each fluid was tested separately or as a pool of 5-9 eggs obtained from the same passage. No constant difference between control and Hodgkin's disease amniotic fluids was noted.

Because patients with Hodgkin's disease may show a debilitating effect which often seems to be much greater than the actual extent of the tumor involvement, the possibility existed of a toxic effect of the Hodgkin's disease agent similar to that present in LGV (Rake and Jones [16]). Yolk sacs from routinely inoculated control and Hodgkin's disease eggs were harvested after a 6-day incubation period. Suspensions were prepared by grinding with sterile sand or by shaking at 187 oscillations per minute for 1 hour; 0.5-ml amounts were injected intravenously into series of mice which were examined for symptoms after 2 and 4 hours and 1, 2, 3, and 4 days. At the end of 4 days all mice were still alive and healthy.

Hemagglutination and Related Techniques

Hemagglutination of erythrocytes by viral agents and the neutralization of the causative hemagglutinins by immune serum were first demonstrated by Hirst (10) in his work with influenza. Since that time many other viruses have shown this property when grown in fertile chicken eggs, either in the extra-embryonic fluids or in a suspension of the ground fetal membranes. Further-
With the hemagglutination by Hodgkin's disease amniotic fluid material apparently not demonstrable, attention was then directed to a study of the effect of human sera, either Hodgkin's disease or control, on the hemagglutinative capacities of other viruses, particularly mumps, vaccinia, NDV, influenza PR8, and Lee. The sera used were from normal and Hodgkin's disease humans and normal and Hodgkin's disease-injected chickens.

To determine any sensitization or stabilizing effect that the Hodgkin's disease amniotic fluid might have on erythrocyte hemagglutinative properties, a series of tests was set up in which erythrocytes were added to undiluted amniotic fluid containing one of the following agents: PR8, Lee, NDV, mumps, vaccinia, or Hodgkin's disease. These erythrocytes were then incubated at 37° C. for 2 hours with frequent shaking (to elute the virus if possible), centrifuged, and the resultant "sensitized" cells resuspended in normal saline. This "sensitized" cell suspension was then added to serial dilutions of Hodgkin's disease amniotic fluid or normal or Hodgkin's disease serum, and readings were made for the presence or absence of hemagglutinations after 1 or 2 hours at room temperature (Table 4). Hodgkin's disease-sensitized erythrocytes were also checked against serum from infectious mononucleosis patients (Table 5). As shown in these tables, no differences could be demonstrated between control and Hodgkin's disease material.

**Table 3**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Chicken erythrocytes</th>
<th>Human erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>Results</td>
</tr>
<tr>
<td>Newcastle disease virus (NDV)</td>
<td>3</td>
<td>Neg.</td>
</tr>
<tr>
<td>Mumps virus (MEV)</td>
<td>2</td>
<td>Neg.</td>
</tr>
<tr>
<td>Influenza (PR8)</td>
<td>2</td>
<td>Neg.</td>
</tr>
<tr>
<td>Influenza (Lee)</td>
<td>2</td>
<td>Neg.</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>2</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Human erythrocytes</th>
<th>Chicken erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amniotic fluid</td>
<td>Hodgkin's disease</td>
</tr>
<tr>
<td>Tests</td>
<td>Results</td>
</tr>
<tr>
<td>NDV</td>
<td>Tests</td>
</tr>
<tr>
<td>1</td>
<td>Neg.</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>1</td>
</tr>
<tr>
<td>Mumps</td>
<td>1</td>
</tr>
<tr>
<td>PR8</td>
<td>1</td>
</tr>
<tr>
<td>Lee</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 5**

<table>
<thead>
<tr>
<th>Human erythrocytes</th>
<th>Chicken erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin's disease human serum</td>
<td>1</td>
</tr>
<tr>
<td>Hodgkin's disease injected chicken serum</td>
<td>1</td>
</tr>
<tr>
<td>Infectious mononucleosis human serum</td>
<td>1</td>
</tr>
</tbody>
</table>

The sera were usually used in a raw state, but in some instances were inactivated at 56° C. for 30 minutes. Either chicken or human erythrocytes were used. Doubling serial dilutions of serum plus 4 hemagglutinative units of one of the above-named viruses, or doubling dilutions of that virus plus 1 serum, were incubated at 37° C. for 45 minutes followed by room temperature for 15 minutes. (Other variations of time and temperature showed no advantage over the standard procedure.) Following this initial incubation period, 0.76 per cent erythrocytes were added and allowed to settle for 1 or 2 hours, depending upon the species of erythrocytes used, before the degree of hemagglutination was recorded (Table 3).
of some of the standard procedures of flocculation, precipitin or complement fixation. The last, particularly, has been used to a great extent as a diagnostic aid in viral and rickettsial diseases. Preliminary surveys have been conducted in an attempt to demonstrate any obvious antigenic reaction by Hodgkin's disease amniotic fluids.

In the slide flocculation test a procedure was used which was similar to that of the Kline-diagnostic test for syphilis. One per cent cholesterylized alcohol (0.5 ml.) was added to an equal amount of 0.85 per cent saline. This suspension was shaken vigorously for 1 minute, 0.8 ml. of pooled Hodgkin's disease amniotic fluid was added and mixed, and 0.5 ml. (0.85 per cent) saline added to bring the final emulsion to an optimum density. This was reshenk less vigorously for 1 minute, incubated at 37° C. for 15 minutes, added to 0.05 ml. serum on ringed slides in amounts of approximately 0.008 ml. followed by 4 minutes of rotation. When checked with this antigen, raw sera gave no reaction at all; inactivated sera showed a nonspecific clumping of particles.

Precipitin studies consisted of the careful laying of 0.1 cc. pooled Hodgkin's disease amniotic fluid (1:5 dil.) over 0.1 cc. serum (undiluted or 1:2), in 95 X 5 mm. tubes. Sera used were from control and Hodgkin's disease-injected chickens, Hodgkin's disease-injected and normal humans. Readings were made after 20-, 60- and 120-minute incubation at 37° C. No reaction could be noted by macroscopic observation in the series of chicken sera nor any difference between Hodgkin's disease and control human sera.

Complement fixation tests have been performed and are being thoroughly investigated. The techniques of Bengtson (1) has been followed, and the reagents have consisted of commercial lyophilized complement and amboceptor, fresh sheep erythrocytes, and inactivated human sera. When collected, these data will be presented in a later article.

Sensitivity (cutaneous).—Intradermal sensitivity tests have become fairly well established as diagnostic procedures, particularly the Frei test for LGV, but also with influenza (Beveridge and Burnet [2]), vaccinia (Smith [18]), mumps (Enders et al. [6]) and herpes simplex (Rose and Malloy [17]). In these various tests a wide range of reactions was noted, with from 54 per cent to 93 per cent of patients with known histories of infection giving positive results.

Amniotic fluid from Hodgkin's disease and control series of embryos 8–15 days old were carefully harvested, pooled, checked for bacterial contamination, and the protein level was determined to be less than 200 mg. per cent. The material was inactivated by heating to 70° C. for 15 minutes. In some instances this was followed by 4 minutes' ultraviolet radiation at 15 inches. The addition of 1:10,000 Merthiolate was discontinued after several mercury-sensitive individuals were encountered. Injections of 0.1-cc. amounts of several such antigens were administered intradermally into the forearms of a group of Hodgkin's disease and non-Hodgkin's disease patients ranging in age from 1 to 79. The results demonstrated only a variable and slightly increased sensitivity of Hodgkin's disease and of some control patients to Hodgkin's disease antigen. This was too irregular in frequency and too slight a degree of response to represent a promising avenue of study.

Animal inoculations.—Repeated attempts have been made in the past to inoculate various animals with Hodgkin's disease lymph node extract. In the present survey it was felt that further study was indicated with the use of Hodgkin's disease amniotic fluid. On the assumption that the agent might have become chicken-adapted through repeated egg passage and, in order to rule out nonspecific foreign protein effects, most of the experiments were done with chickens. Two separate series were set up. The first consisted of six adult chickens, three inoculated with Hodgkin's amniotic fluid and three with control amniotic fluid. Intramuscular inoculations were made over a period of approximately 2 months, the dosage increasing from 0.5 to 1.5 cc. The Hodgkin's disease material was originally from four patients, the control from five. At autopsy, 2 or 3 months after the first injection, the tissues were carefully examined grossly and microscopically, and, when possible, blood was obtained for serologic study. A second experiment, consisting of only one chicken in each group, was set up at a later date. No distinction could be made in either series with regard to tissue changes or to serologic reactions in various hemagglutination tests referred to above.

Attempts to demonstrate development of intradermal sensitivity were made during the study of the effects of Hodgkin's disease inoculation. Chickens, guinea pigs, rabbits, rats, hamsters, and monkeys were used. Hodgkin's disease and control material, consisting of human lymph node cell-free extract, was injected into separate series of animals, the volume varying from 0.1 cc. to 0.3 cc. according to the size of the animal, and repeated 4–6 times. After an interval of 10 days, 0.1-cc. amounts of amniotic fluid from appropriate Hodgkin's disease and control cases were injected intradermally. The intensity of the reaction and actual diameter of any wheals were carefully determined after 24 hours. In some instances the guinea pigs showed a
nonspecific reaction, and results were inconclusive in regard to the rats. All other species showed no response whatever to the Hodgkin's disease material.

Among other animal experimental work, one series of seven experimental and seven control young adult mice was inoculated intracerebrally. They developed no signs and symptoms and upon post mortem examination showed no histologic changes. Attempts to produce corneal lesions were also unsuccessful.

Interference.—It has been established by many workers (Price [15], Vilches and Hirst [19], Ginsberg and Horsfall [7]) that in certain circumstances the presence of one virus will interfere with the growth of a subsequently inoculated one. In some instances a definite order of inoculation must be followed, while others inhibit one another regardless of the order of inoculation. The time interval between inoculations is often very important and varies with each combination of viruses.

Following preliminary surveys in which Hodgkin's disease was used as the first inoculum and various known viruses, including PR8, Lee, NDV, mumps, and LGV as the second, a large series have been run in which Hodgkin's disease fluid from 12-day live, fertile chicken eggs was inoculated into 7-day embryos (amniotic sac) followed 3 days later by Lee virus (Bostick [4]). The amniotic fluid was harvested 18 hours later and titered for the presence of Lee virus by the determination of its hemagglutinins, with human erythrocytes. Control materials tested have included normal amniotic fluid, as well as control material from non-Hodgkin's disease malignancies. Both Hodgkin's disease and control materials were carried through at least four egg passages before being tested. All materials were stored in a deep-freeze (—20° C.) refrigerator between passages. The results are shown in Table 6 below. The degree of interference of the growth of the Lee virus by the Hodgkin's disease agent was variable. Sometimes there was complete absence of the virus, as demonstrated by both hemagglutination and infectivity tests. On other occasions it was manifest by a decreased hemagglutination titer. This phenomenon was demonstrable not only by the amniotic fluids from eggs inoculated originally with lymph nodes ground and pooled from several Hodgkin's disease patients, but by those from individual Hodgkin's disease patients. This capacity to interfere with the growth of the viruses will provide a greatly simplified tool for the study of the Hodgkin's disease-isolated agent.

**DISCUSSION**

The application of a new but established technic to an old problem may result in the discovery of important facts which go far toward the solution of the problem. Hemagglutination phenomena were studied from many aspects. The Hodgkin's disease amniotic fluid was not hemagglutinative for any of many types of erythrocytes. It was also incapable of altering or “sensitizing” erythrocytes so that they would possess aberrant hemagglutination responses to most of the known hemagglutinative viruses. The Hodgkin's disease factor could not, therefore, be detected by these methods.

The Hodgkin's disease amniotic fluid was tested for an immediate toxic effect in various ways. Intravenous inoculations into adult mice failed to demonstrate any immediate or delayed toxic or infective effect of the material. Such animals are very sensitive to certain known viruses when administered in that manner.

In the chicken embryo, in addition to the mildly toxic effect of the Hodgkin's disease amniotic fluid, there is a tendency for an increase in the protein level of the amniotic fluid. However, this was of questionable significance and was not reflected in any abnormal change of weights of these embryos.

Most of the standard serological methods have failed to demonstrate any specific characteristic in the Hodgkin's disease amniotic fluid. Precipitin tests were negative, as were flocculation tests modeled after the Kline technic. Complement fixation methods were the only ones which showed some trend of specificity, and further data are being collected on them.

In agreement with the observations of many preceding investigators, it was impossible to cause any changes in many kinds of experimental animals upon inoculation with Hodgkin's disease lymph nodes emulsion or Hodgkin's disease amniotic fluid material. No intradermal sensitivity to this material could be detected in the injected animals.

The best approach for further exploration is the study of virus interference phenomena. Since the Hodgkin's disease agent itself possessed no easily identifiable effect on the chicken egg, its ability to interfere with the growth of a virus known to possess characteristic properties was investigated.
Several viruses were studied according to their ability to grow in fertile chicken eggs previously inoculated with the Hodgkin’s disease agent. The growth of Lee influenza virus was noted to be susceptible to interference by the Hodgkin’s disease material; initial inoculation depressed or completely interfered with the ability of the Lee virus to grow. Its growth was judged by hemagglutinative titer methods and, on occasion, by egg-infectivity titers. Careful parallel controls were run at all stages. This reproducible and quite rapid test for the presence of the Hodgkin’s disease amniotic fluid material will greatly aid in the further study of Hodgkin’s disease.

The Hodgkin’s disease amniotic fluid harvested following numerous serial egg passages is most satisfactory for study by certain physical-chemical procedures. This material is now being studied by electron microscope, ultracentrifuge, and differential precipitation techniques.

**SUMMARY**

The possible infectious characteristics of Hodgkin’s disease have been re-examined by the previously unused technic of serial passage of the Hodgkin’s disease lymph node extracts in embryonated chicken eggs. A slight lethal effect in the embryos was noted. The harvested amniotic fluid was shown to possess virus growth interference activities against Lee influenza virus grown in chicken eggs.

This harvested amniotic fluid was examined for other properties. No hemagglutinative tendencies were encountered when tested against erythrocytes from many animals. No sensitization effects on erythrocytes later exposed to known hemagglutinative viruses were noted. The amniotic fluid had no untoward effect upon animal inoculation via many routes. Precipitin and flocculation tests were uninformative, and complement fixation procedures showed some trends that deserve further study.

The amniotic fluid harvested after numerous serial passages from Hodgkin’s disease-inoculated embryos has been shown to possess certain filterable, transferable, and virus-like properties. This material should make possible extensive studies by serological, chemical, and physical methods.

**REFERENCES**

Experimental Studies on the Etiology of Hodgkin's Disease

Warren L. Bostick and Lavelle Hanna


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/11/7/505

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.