The evolution of the antibody, as a γ-globulin variant of the physiologically active molecule, endows the animal with a defense mechanism of considerable survival value. There would reasonably follow a selective evolutionary advantage and a further strengthening of the defense mechanism. It is such a variant, of epidermal hair, bunched together on the nose of a rhinoceros, that finally culminated in its present and effective nose horn.

Summary

A physiological role of γ-globulin has been established for human and dog erythrocytes and polymorphonuclear leucocytes. Specific γ-globulin binds to the membrane of the cells and determines their conformation, viability and function. The spleen appears to be primarily responsible for the synthesis and excretion of these γ-globulin molecules.

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Blood-Borne Virus-Like Agents in Hodgkin's Disease, other Malignancies, and Systemic Lupus Erythematosus

Results of a Large Scale Survey by Means of a New Serological Screening Test

S. E. Moolten, E. Clark, M. Mihalyfi *

Lymphology 1 (1970), 31–50

The studies reported here embody a new principle in technical methods for detection of hidden viral infection as applied to certain disease categories. The technique is based on the long known observation that the red blood cell can adsorb to its surface a large variety of viruses (1) and virus-like agents and may thereby become the vehicle of their transport in the circulating blood (2). Red blood cells circulating innumerable times through diseased tissue may be held to pick up with each successive passage an additional "load" of viral material. An illustrative parallel may be drawn with the selective affinity of certain ion-exchange reins for specific non-living molecules. Repeated perfusion of the tissue with the same blood cells multiplies their chances of accumulating a rela-

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tively high surface concentration of free virus. At this stage the virus may elute spontaneously into the serum or into a selected suspending medium which can be inoculated without further treatment into chick embryos, tissue cultures, or other virus media.

From our experience with this technique it appears that the use of the patient's blood as the inoculum has certain advantages over use of the diseased tissue itself as a source of virus or virus-like agents. In addition to its simplicity the method provides an inoculum which is relatively unencumbered with toxic cytoplasmic and nuclear debris or contaminating microorganismus such as bacteria or fungi harbored by the source tissue.

The binding property of red cells for myxoviruses was shown by Burnet and his group (1c) to condition the cells to the agglutinating action of antiviral sera. This principle was applied in our earlier studies (3) to the development of a serological procedure adapted for mass screening of patients for various blood-borne virus-like agents. The procedure is similar in principle to that of the tanned red blood cell hemagglutination technique and techniques in which antigens adsorbed to particulate vehicles (Collodion particles, Latex particles, Bentonite, etc.) are agglutinated by the corresponding immune sera. In our procedure the patient's red blood cells, regarded as the antigen-carrying particle, are tested against a panel of specific antiviral sera.

The data reported here comprise the combined results of two large series of tests, the first conducted between 1950 and 1953 (*S. E. Moolten* and *Ellen Clark* with the collaboration of *Anthony Rottino*) and the present one conducted since 1965 (*S. E. Moolten, Miklos Mihalyfi,* and *George Hyman*). In the selection of case material for this study the writers gave particular attention to disorders associated with abnormally rapid destruction of red blood cells. These included acute hemolytic anemias of unknown cause, both Coombs' positive and Coombs' negative, and hemolytic anemias occurring as complications of Hodgkin's disease, chronic lymphatic leukemia, infectious mononucleosis, systemic lupus erythematosus, and certain other disorders. The initial study was quickly extended to include cases of the latter group of diseases without reference to the presence or absence of overt hemolysis on the supposition that milder degrees of hemolytic disorder might be masked by compensatory increase in red cell production.

Methods

A. *Isolation of Virus-Like Agents in Chick Embryos*

Red cells and serum were obtained from samples of the patient's venous blood which had been allowed to clot at room temperature. The inoculum consisted of 0.1 cc. of the serum in which the red cell concentration was approximately 0.5 per cent. The red cells selected for inoculation were those which had sedimented to the bottom of the tube of clotted blood. Penicillin and streptomycin were added to the suspension, which was then inoculated into several chick embryos 10 to 11 days old, using the chorioallantoic membrane (CAM) and in some instances the amniotic cavity or both.* The eggs were incu-

* The eggs were of certified stock, obtained in the initial studies from the N. J. Agricultural Experiment Station (Rutgers University) through the kindness of Prof. *John Pino* and in all subsequent studies from Vincent Darago. In the experience of one of us (N.M.) with the latter source of embryonated eggs during several years of virus research at the Institute of Microbiology, Rutgers University, they have been uniformly free of avian viruses or mycoplasmas.

bated at 36 °C for 2 or 3 days or longer. The movements of the embryo were watched twice daily and after an appropriate time the eggs were opened and the CAM was studied for virus-type plaques. The allantoic fluid was tested for hemagglutinating activity against chick embryo erythrocytes and a pool of allantoic fluid from several infected embryos was collected for immunizing several rabbits (usually 4) against each particular strain of virus-like agent.

Culture and smear techniques were employed routinely to exclude the possibility of bacterial infection. In the early study of isolates their filter-passing ability was established according to standard techniques. Through the kindness of Prof. *R. L. Reagan* the virus-like nature of an isolate from a patient with Hodgkin's disease was further demonstrated by means of electron micrography, which disclosed the presence of spherical particles averaging 75 mμ in diameter (the approximate size of an adenovirus particle). Histological examination of infected embryo membranes and other tissues was performed in selected cases. (Pending further study to verify the viral nature of these agents and, where possible, their other biological characteristics and molecular structure the writers hold it advisable to refer to them by the general term "virus-like agents".)

B. *Preparation of Rabbit Antisera*

A panel of specific immune sera was produced in rabbits against virus-like agents isolated from the blood of patients with Hodgkin's disease, systemic lupus erythematosus, and infectious mononucleosis. Healthy 3 kg rabbits were injected intramuscularly with 2 cc. of infected allantoic fluid emulsified with Freund's adjuvant. This was repeated at 4 weeks. Samples of rabbit serum were tested at intervals for specific antibodies by neutralization and hemagglutination-inhibition techniques. Booster doses of allantoic fluid were given in amounts of 1 cc. intraperitoneally or intramuscularly without adjuvant until a fairly high titer of antibodies was obtained. Before use the rabbit serum was inactivated at 56 °C for 30 minutes and adsorbed against pooled normal human red cells of all available types in the hospital's Blood Bank to remove species-specific and blood group-specific antibodies. Control sera employed in the first series (1950-1953) consisted of antisera against mumps, Newcastle disease, influenza, lymphocytic choriomeningitis, Arbo viruses, psittacosis and brucellosis. In the current series normal rabbit serum and saline were employed as controls.

C. *Agglutination Technique for Virus-Conditioned Red Blood Cells (Method of Moolten and Clark (3b)*

A sample of the patient's venous blood was allowed to clot at room temperature. Red cells freed from the clot were washed lightly and then added in 2% saline suspension to serial two-fold dilutions of each of the immune sera of the panel. The tubes were observed after 2 hours for specific agglutination of typical antigen-antibody type, stable at 37 °C (Fig. 1).

Results

Embryos infected with virus-like agents isolated in the disorders under study exhibited typical „virus" plaques on the chorioallantoic membrane (CAM). These were usually evident within 72 hours on primary inoculation with serum-red cell samples from patients with infectious mononucleosis and SLE. In the case of Hodgkin's disease the

plaques were not visible until at least 96 hours had elapsed and often failed to make an appearance before the second egg passage. The plaques in infectious mononucleosis were minute (as also described in a similar study by Nettleship (4); those in SLE were larger and denser (Fig. 2). In Hodgkin's disease the plaques were slower to form, flatter, and more translucent than in the case of SLE.

Isolation of virus-like agents was accomplished in approximately 1 out of 10 attempts, the effort tending to be more often successful in the earlier phases of the patient's illness or during flareups of disease activity. The results of tests with immune sera for red cell attachment of virus were not always correlated with positive isolations of the agent, in certain instances growth being obtained in embryos when red cell agglutination tests

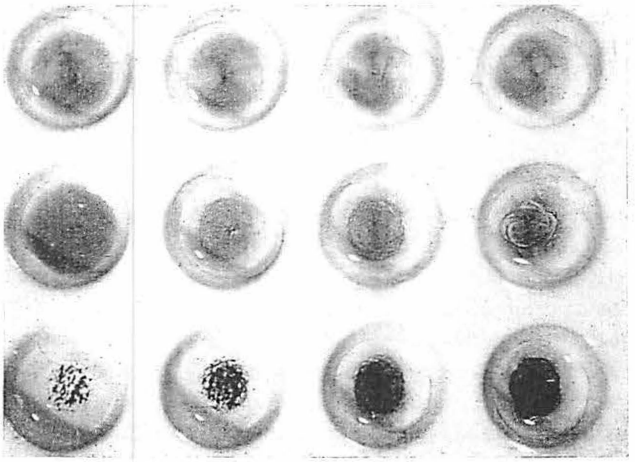

Fig. 1 Serological identification of red cell-adsorbed virus by specific antiserum. Positive agglutination test (1 : 20) with specific antiserum (lower row). Negative results with control serum and control red cells (upper 2 rows).

were negative and vice versa. (Studies are now in progress in search of a method for preventing elution of virus from red cells into serum in an effort to increase the sensitivity of the agglutination technique.)

The results of the agglutination studies with antiviral sera are comprised in two bodies of data, one accumulated in the period 1950 to 1953, the other 1965 to the present. The test samples of blood of the first series were obtained in part from Dr. *A. Rottino,* Director of Laboratories, St. Vincent's Hospital, New York City, in connection with his own studies of Hodgkin's disease. These samples (a total of 341) were brought to us in code-numbered tubes without identification of their source from patients with Hodgkin's disease, other disorders, benign or malignant, or healthy controls until after the test results had been made known to Doctor *Rottino.* An approximately equal number were obtained from patients treated at St. Peter's Hospital, New Brunswick, where earlier studies were being conducted. After a hiatus of several years, the studies were resumed in March 1965 and conducted in similar fashion at Middlesex General Hospi-

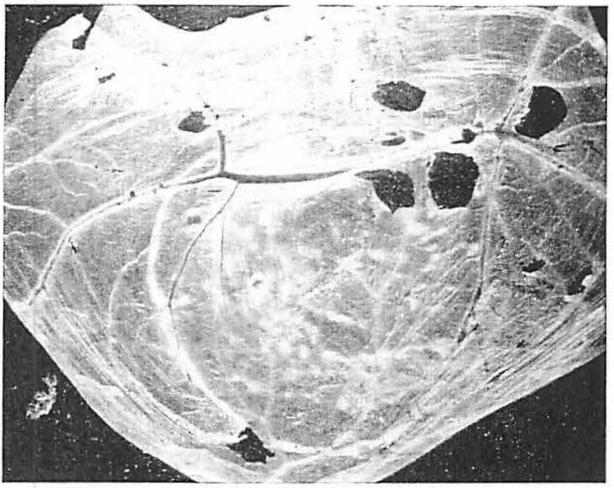

Fig. 2 A Virus-type plaques produced by agent isolated from blood of patient with systemic lupus erythematosus (serial passage, chick embryo CAM).

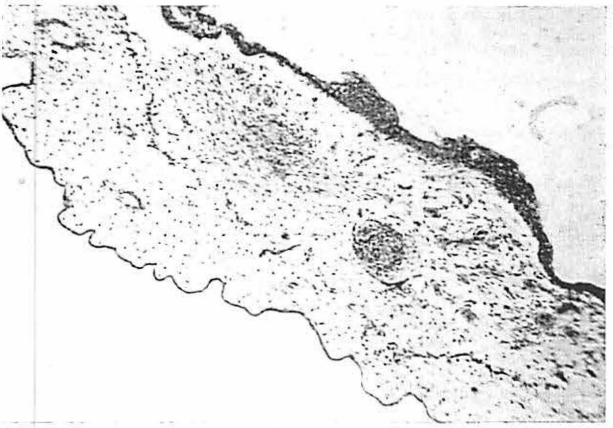

Fig. 2 B Ectodermal thickening and mesodermal nodules in CAM (low power photomicrograph).

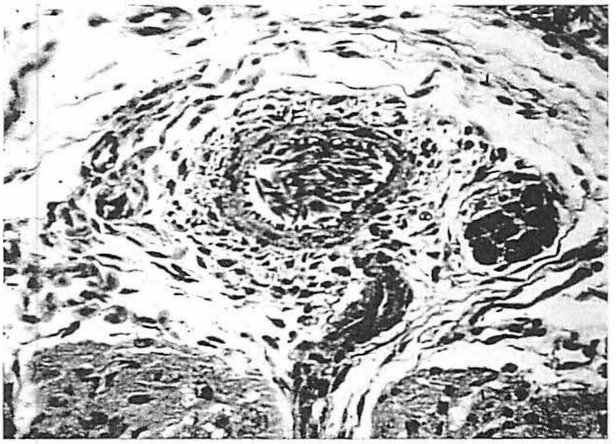

Fig. 2 C Perivascular infiltration of inflammatory cells (epicardium of chick embryo).

tal, New Brunswick. All samples tested in the recent series were obtained from Dr. *George Hyman,* Hematologist of Francis Delafield Hospital, Columbia University, New York City.

Results of Tests with „Hodgkin's Disease" Antiserum (Antiserum against Agents Isolated in Cases of Hodgkin's Disease): As already indicated, two series were studied separated by an interval of 12 years. Both series were conducted with careful attention to controls. Observer bias was prevented by withholding the name and diagnosis of the donor of the blood sample until after the results had been reported. The specificity of the immune sera was controlled by the use of a large panel of test sera including normal rabbit serum and saline. Results were listed as positive only when agglutination was unmistakably present in serum dilutions of 1 to 5 or higher. In the first series the immune serum was produced in rabbits with a single strain of virus-like agent (A. J.) isolated in a case of Hodgkin's disease. In the second series (1965-1968) immune sera were produced in rabbits with strains of virus-like agent isolated from 6 patients suffering from Hodgkin's disease (JN, DS, KT, N, D, R). The 6 sera were shown to possess close antigenic relationship by the results of cross-neutralization tests in infected chick embryos, indicating the probability that we were dealing with a single virus species.

The single "HD" antiserum employed in the first series (table 1) gave positive results in one third of the tests (32 out of 96) with blood cells from patients with Hodgkin's

Table 1 Red cell agglutination with "HD" immune serum. (Antisera against virus-like agent [AJ] isolated in Hodgkin's disease, 1950–1953.)

	Positive Results	Total Tests
Hodgkin's Disease	32	96
Leukemia – LSa	7	33
Carcinoma	4	20
Other Diseases and Normal Subjects	7	524

Table 2 Red cell agglutination with "HD" immune sera. (Antisera against virus-like agents JN, DS, KT, N, D, R, isolated in Hodgkin's disease, 1965–1968.)

	Positive Results	Total Tests
Hodgkin's Disease	62	537
Leukemia – LSa	17	437
Carcinoma	6	117
Other Diseases	2	259
Normal Subjects	0	63

disease contrasted with positive results in less than 2 per cent of a large number of controls. In the second series (table 2) the proportion of positive results was considerably lower (62 out of 537). Positive results were obtained in several cases early in the course of the disease but were uniformly negative subsequently.

In both series positive results with the "HD" serum were obtained in a number of tests of blood obtained in cases of leukemia, lymphosarcoma, and a wide range of carcinomas, although in distinctly lower ratio to the total number of tests than in the case of Hodgkin's disease. For example in the first series (table 1) 7 out of 33 tests in cases of leukemia or lymphosarcoma were positive and 4 out of 20 tests in cases of carcinoma. In the second series (table 2) 17 out of 437 tests in cases of leukemia or lymphosarcoma (including myeloma) were positive and 6 out of 117 tests in cases of carcinoma (breast, lung, prostate, esophagus, colon, and other organs). Only 2 tests were positive out of

259 tests in cases of non-malignant disorders (1 case of severe cirrhosis, 1 case of congestive heart failure). All normal subjects tested uniformly negative.

The antigenic relationships between the 6 separate "HD" strains was demonstrated not only by the cross-neutralization tests in chick embryos but also by the tests in human subjects with the same antisera. In series II, comprising 53 patients suffering from Hodgkin's disease and a large number of patients with other disorders and healthy controls tested one or more times, positive results were obtained with more than one

Table 3 Comparative incidence of positive results with 6 "HD" sera in patients with Hodgkin's disease.

No. Test Samples per Case	No. Cases	No. Positive	No. Sera giving Positive Results					
			6	5	4	3	2	1
1	19	3					1	2
2	12	5			1			4
3	6	3				2	1	
4	5	3				2	1	
5	2	1				1		
6	1	1						1
7	1	1						1
8	3	2					2	
9	1	1						1
11	1	1						1
12	1	1					1	
18	1	1				1		

"HD" serum simultaneously or at different times (table 3, table 4). No significant differences could be distinguished between the antigenicity of the strains for rabbits nor in the relative proportion of positive results obtained by any of the sera tested against the blood samples.

Results of Tests with "IM" Antiserum (Antiserum against Agent Isolated in Cases of Infectious Mononucleosis) (table 5): For purposes of comparison and as an additional control several sera were prepared in rabbits against the virus-like agent isolated from the blood of patients with infectious mononucleosis and were included in all test panels. In the first series the test results of cases of infectious mononucleosis, presenting typical changes in leukocytes and positive heterophile reaction, were positive with the "IM" antiserum in 45 out of 58 tests (77,6%) in contrast to 12 out of 363 controls (3,3%). The incidence of positive tests was somewhat higher than in the controls in cases of malig-

Table 4 Comparative incidence of positive results with 6 "HD" sera in other disorders*.

Diagnosis	No. of "HD" Sera giving Positive Results					
	6	5	4	3	2	1
Chronic Lymphocytic Leukemia		–			1	1
Lymphosarcoma				2	2	1
Chronic Myeloid Leukemia					1	
Acute Leukemia						2
Myeloma						2
Carcinoma					1	4
Cirrhosis						1
Congestive Heart Failure						1

* Number of repeat tests in same patient unknown.

Table 5 Red cell agglutination with immune sera against virus-like agent of infectious mononucleosis.

Disease	Series I (1950–1953)		Series II (1965–1968)	
	Positive Results	Total Tests	Positive Results	Total Tests
Hodgkin's Disease	3	63	2	178
Leukemia – LSa	4	26	4	145
Carcinoma	0	5	3	51
Systemic Lupus Erythematosus	0	60		0
Infectious Mononucleosis	45	58		0
Other Diseases and Normal Subjects	12	363	1	124

nant disorders (Hodgkin's disease, leukemia, lymphosarcoma, and carcinoma), 7 out of 94 such cases being positive (7,4%). In series II the incidence in malignant disorders was 2,4%, in non-malignant disorders and normal subjects less than 1% (1 out of 124). No cases of clinically active infectious mononucleosis were available for test in the second series.

Results of Tests with "Lupus" Antiserum (Antiserum against Agents Isolated in Cases of Systemic Lupus Erythematosus): The results of tests with antiviral serum produced against a virus-like agent isolated in cases of SLE were striking for their high specificity. As indicated in table 6, 34 out of 60 tests with this serum were positive in cases of proved SLE, all yielding a positive test for the LE factor. In several cases strains of a serologically homogeneous species of virus-like agent were isolated from the blood in chick embryos (Fig. 2). The results of the serological test of red cells were uniformly

Table 6 Red cell agglutination with immune serum against virus-like agent of systemic lupus erythematosus.

Disease	Series I (1950–1953) Positive Results	Series I (1950–1953) Total Tests	Series II (1965–1968) Positive Results	Series II (1965–1968) Total Tests
Hodgkin's Disease	0	95	0	178
Leukemia – LSa	0	33	0	145
Carcinoma	0	20	0	51
Systemic Lupus Erythematosus	34	60		0
Infectious Mononucleosis	0	38		0
Other Diseases and Normal Subjects	0	271	0	124

negative in 148 tests in cases of malignant disorders and in 309 tests in cases of other nonmalignant disorders and controls. In Series II the results were negative in all cases of malignant disorder (374 tests) and nonmalignant disorders or controls (124 tests).

One additional observation of interest was the *negative* results obtained with „IM" antisera in the presence of positive results with "HD" antisera. Conversely in cases of Hodgkin's disease and other malignancies in which positive results were obtained with "HD" antisera on one or more occasions the tests were negative with the same antisera at the time such patients gave positive results with "IM" antiserum. None of the samples tested in cases of clinical infectious mononucleosis, regardless of the result with "IM" antiserum, was positive at any time with "HD" or "LE" antisera. Similarly none of the cases of systemic lupus erythematosus, with or without positive result with "LE" antiserum, was positive at any time with "HD" or "IM" antisera. Not a single case in the entire study gave a positive result with "LE" antiserum except cases of typical systemic lupus erythematosus with positive LE phenomenon.

Discussion

The capability of the red blood cell to bind antigenic material to its surface has been widely exploited as an immunologic tool. To the virologist this property of the red cell has proved invaluable in the detection and serological classification of a large number of viruses by in vitro methods.

The Red Cell as a Vehicle of Virus Dissemination

Shwartzman (2) suggested the mechanical role of red cells in transporting the virus of lymphotic choriomeningitis in mice from portal of entry to target tissue and possibly other viruses which are similarly pantropic. *Downie* and his associates (5) thought that the virus of smallpox in man may be disseminated by the cells of the blood rather than by serum, and *Hamre* (6) found evidence for the red cell as a carrier of influenza virus in mice. *Overman* (7) found the same in the case of mumps with viremia and also noticed that the residual red cells of the blood clot which had settled in the serum showed a "typical viral hemagglutination pattern" (1958). This was the original reason for his idea of testing

blood for viremia. *Kilham* (8) in 1948 had the same idea. As might be anticipated, viremia in mumps, smallpox and similar conditions is of short duration and unlikely to persist after the appearance of immunity.

Viremia as a Factor in Hemolytic Anemia: The possibility that overwhelming viremia occurring in infection with a hemolyzing virus may give rise to severe hemolytic anemia was the subject of our initial encounter with this problem in the case of a patient whose

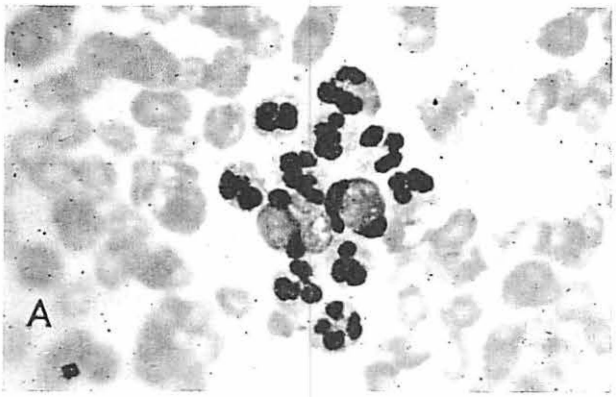

Fig. 3 A LE cell phenomenon induced by incubating normal human buffy coat with virus-like agent isolated from blood (patient with systemic lupus erythematosus).

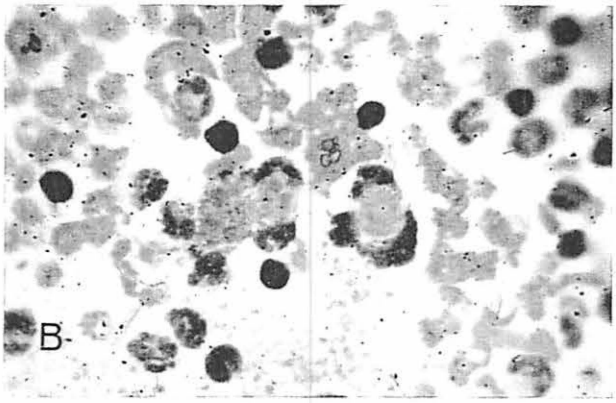

Fig. 3 B LE cell phenomenon induced in guinea pig leukocytes with same agent.

blood contained a high titer of Newcastle disease virus (3a). This experience drew our attention to the role of blood-borne viruses in the pathogenesis of anemia complicating a variety of other disorders such as systemic lupus erythematosus and Hodgkin's disease. We were fortunate in having available for study two cases of systemic lupus erythematosus complicated by severe anemia and leukopenia. A virus-like agent with hemagglutinating properties was isolated in chick embryos from the blood of both patients. The allantoic fluid of the infected embryos was found capable of inducing the typical LE phenomenon when incubated with normal buffy coat leukocytes of human and guinea pig origin and of maintaining this capacity through many chick embryo passages (Fig. 3). Further studies in the case of Newcastle disease virus infection proved interesting in the fact that the patient eventually developed a high titer of anti-NDV antibodies during

her convalescence from acute hemolytic anemia and coincidentally a positive Coomb's reaction on test of her red cells. Her serum contained no antibodies for normal red cell but contained antibodies which attached to normal red cells following their exposure to Newcastle disease virus in saline as demonstrated by positive Coomb's test (3b). This was the essential observation which provided the conceptual basis for our present method of testing red cells of patients for adsorbed virus with specific immune sera.

The Immunological Significance of Chronic or Recurrent Viremia

When a virus-like agent persists in the blood, as revealed by repeated successful isolation of the agent in chick embryos and repeated positive results of serological tests of red cells for adsorbed viral antigen, interest is naturally aroused concerning the competence of the affected patient's immune mechanisms. The presence of prolonged or recurrent viremia compels one to assume either that the agent recovered from the patient's blood lacked antigenicity or that the patient's immune processes were incompetent against that particular agent. That the former assumption is unlikely is suggested by the regularity with which all the blood-borne agents examined by us thus far were capable of inducing sharp rises in antibody titer upon injection into rabbits. Their antigenicity for the human was also demonstrated in the case of a healthy volunteer, the husband of one of the patients with systemic lupus. After repeated injections of formalin-inactivated "LE" virus he developed a rising titer of neutralizing antibody against the "LE" virus-like agent and his serum inhibited the LE cell phenomenon when mixed with the patient's serum or virus. Injections of his plasma intramuscularly into the patient appeared to induce temporary partial clinical remission and disappearance of the LE phenomenon in tests with her blood (3b).

Viral Transfer In Utero in Relation to Chronic or Recurrent Viremia and The Concept of "Vertical" Transmission of Disease: It has become increasingly apparent that circulating antibodies are relatively unimportant in checking viral multiplication and limiting its spread within the tissues (9). Evidence now available indicates that cells of the monocytic series become sensitized to viral antigens and that resistance against viral multiplication may depend primarily on the lymphocytes. Thymectomy, for example, increases the oncogenic effect of the polyoma virus in mice (10). Immune responses which may appear following infection with certain viruses, particularly such oncogenic viruses as SV 40, polyoma, and adenovirus Types 12 and 18, whether in the form of humoral antibodies or a cellular reaction of the homograft-rejection type, may represent the development of new cellular antigens arising out of the "temperate" virus-cell relation (9, 11).

The example of lymphocytic choriomeningitis virus infection of mice is relevant in this connection. Mice infected with LCM virus *in utero* or in early infancy have been found to possess remarkable tolerance for the virus despite the fact that virus was present in practically every organ as well as in blood, urine, and nasal secretions. Although the infection in these mice was inapparent it could be transmitted to their offspring and to contacts (12). When infection was transmitted to mature mice the virus was recoverable for only a short period and the animals developed strong immunity. These mice were non-infective for other animals.

The model of inapparent LCM virus infection in mice infected *in utero* associated with failure to develop immunity has certain parallels in other species including man. A wealth of evidence is now available to indicate the role of the thymus in determining the capacity of the small lymphocyte to participate in the immune response, whether in the form of the delayed hypersensitivity response to infection or in the rejection of tissue grafts. Inasmuch as this property of the thymus does not become manifest until late embryonic life or early infancy, it becomes possible for intrauterine infection to exist in inapparent form throughout the lifetime of the individual and to be transmitted transplacentally from generation to generation, as in the case of LCM virus infection of mice. A common example in man is the transmission of cytomegalovirus infection in inapparent form either in early infancy or during fetal life. The concept of "vertical" transmission of viral infection exemplified in the case of LCM provides a particularly acceptable explanation for the transmissible leukemias of chickens, mice, and cows, as pointed out by *Gross* (13). Only when the carrier hosts reach middle age do they usually develop leukemia. Whereas animals of any age can be successfully inoculated with the transformed cells of a virus-induced tumor, injections of oncogenic viruses have to be given to animals shortly after birth in order to insure uniform tumor growth. Adult animals can be made more susceptible to such viruses by neonatal thymectomy. On the other hand, newborn mice can be made more resistant after treatment with viral antiserums or after transplantation of isologous lymph node cells from normal adult mice which provide the mechanism for immunologic competence lacking in the newborn animal (14).

The vertical transmission of virus-induced disease is not necessarily confined to tumor-producing viruses. Indeed, the virus of LCM is now known to cause a late onset disease of the glomeruli which may be responsible for the early death of animals infected since birth. The smudging of the glomerular capillary loops without cellular infiltration, as suggested by the illustrations published in a recent paper of *Baker* and *Hotchin* (15), bears certain similarities to the glomerular changes of lupus nephritis. One of the best known animal models of human systemic lupus erythematosus is the disorder occurring spontaneously in a strain of hybrid New Zealand mice (NZB/BL-NZW). The renal lesions in these mice are typical of human lupus nephropathy. They occur in all females and in half the males. Typical LE cells, hemolytic anemia, and periarterial splenic fibrosis appear (16a). Cell-free filtrates induced the development of a similar disorder in 10–20% of hybrid Swiss mice (16b). In addition, virus particles were demonstrated by electron microscopy in the organs of NZB mice. Hyalin glomerular lesions with smudging of glomerular basement membranes and fibrinoid arteritis are also part of the characteristic picture of viral plasmacytosis (Aleutian Disease), a transmissible viral infection of Aleutian mink (17).

Vertical Transmission as a Basis for "Slow Virus Infection": Vertically transmitted viral diseases of these types fulfill the criteria laid down by *Sigurdsson* in 1954 (18) for the group of diseases he named "slow virus infections". These criteria are 1. a very long initial period of latency lasting from several months to several years; 2. a rather regular protracted course after clinical signs have appeared, usually ending in serious disease or death; 3. limitation of the infection to a single host species and anatomical lesions in only a single organ or tissue system. In terms of the possible role of immunological toler-

ance in the slow virus infections *Sigurdsson* stressed that "in chronic infections the immunity mechanism of the body never gets a good grip on the pathogenic microbe and therefore a long and dubious battle ensues".

Familial Occurrence of Hodgkin's Disease, Leukemia, and Systemic Lupus Erythematosus: Should the findings in mouse leukemia and the lupus-like syndrome of NZB mice be applicable to human disease, it would logically follow that in order for the causative viruses to be transmitted from man to man they would have to be introduced before birth; i.e. transplacentally. Some hint of this sequence is suggested by clinical observations of the more frequent occurrence of leukemia, Hodgkin's disease, and systemic lupus erythematosus in certain families. The concurrence of both leukemia and Hodgkin's disease in blood relatives of the same family has also been described by *Gross* (13) and by *Mazar* and *Straus* (19). The latter authors surveyed the literature and uncovered reports of 32 groups of cases of blood relatives with Hodgkin's disease, the majority being siblings or offspring of women with Hodgkin's disease.

Numerous reports also concern the occurrence of multiple cases of systemic lupus in the same family. The data in all the cases available thus far (with one possible exception) indicate that these are generally blood line relatives such as siblings or mother and child (20). The concept of placental transmission of a virus-like agent as the basis for lifetime infection which may either remain latent indefinitely or erupt periodically in mild or severe form and in various clinical guises is wholly compatible with what is known of the protean manifestations of systemic lupus erythematosus.

Flaring Factors in Slow Virus Infection: Certain parallels can be drawn between SLE and herpes simplex infection. The latter is now generally regarded as having its onset in early childhood as an acute illness (acute gingivostomatitis). Thereafter the virus is harbored as a lifelong inhabitant in latent form except when mobilized by such flaring factors as high fever, chilling, severe emotional upset, menstruation, allergic reactions, vaccine injections or excessive exposure to sunlight. A striking similarity exists with the flaring factors which initiate or exacerbate the clinical activity of SLE, e.g. excessive exposure to sunlight (or ultraviolet light), frostbite, burns, severe emotional shock, reactions to gold injections, iodides, atabrine, penicillin, vaccines against diphtheria or scarlet fever, and pregnancy. The oft-repeated hormonal stress of menstruation, long known to cause flareups of tuberculous infection and other chronic infections in young women, may well account for the striking predilection of SLE for females during the active years of menstrual function.

Following the same reasoning, one should logically assume that individuals with obvious tumors or leukemia represent actually a small fraction of subjects actually carrying the seeds of either disease. Triggering stimuli which activate the hitherto masked agent to become formidable pathogens may exist in the form of such external factors as x-rays and other form of radiant energy, certain chemical cell poisons, repeated trauma ("chronic irritation"), and hormonal influences (*Gross* 13).

The activating or unmasking effects of carcinogens on viruses is well known. *Dulbecco* (21) (1955) found that methylcholanthrene activated latent fowlpox virus in chickens when applied to their skin. Intradermal injection of vaccine virus into mice prepared by skin paintings with methylcholanthrene combined with injections of cortisone results in the development of a much enhanced dermal infection which is frequently

followed by a variety of benign and malignant neoplastic lesions (*Duran-Reynals* 22). Polyoma-injected mice which had carcinogenic or tumor-promoting substance applied to the skin developed significantly more polyoma-type tumors than the mice which received the virus alone (23).

The role of interferon in protection against virus-induced tumors and depression of circulating interferon as a factor in activating tumor-inducing viruses should be given serious consideration in this connection. Interferon has been shown to protect animals against the induction of tumors with polyoma virus or Rous sarcoma virus and to inhibit malignant transformation by SV 40 virus in vitro. Administration to mice of urethan, a known carcinogen, decreases the level of interferon in the blood and reduces significantly their resistance to a variety of viral infections, including mouse pneumonia virus, mouse hepatitis virus, and leukemia viruses (24).

The possible role of interferon in the present series deserves further study as suggested by the observation that red cells carrying one virus "charge" appeared to be inagglutinable by antisera against any other type of virus. This was shown for example by the uniformly negative results of tests for the HD agent on the red cells of patients with Hodgkin's disease or other malignancies whose cells reacted with the IM antiserum despite the fact that at other times they reacted positively with the HD antiserum. Another factor to consider is the level of the "heat-labile factor" (properdin) in the serum of these patients. In an early study we encountered three patients with viremia whose untreated serum failed to inhibit hemagglutination of guinea pig erythrocytes with NDV in contrast to the serum of normal subjects (3 b).

Relation of Hemagglutinating Viruses to Hemolytic Anemia: Several observations have been reported indicating that viruses responsible for specific disease entities may also account for complicating hemolytic anemia. In newborn humans the viruses of cytomegalic inclusion disease (25) and rubella (26) may produce, in addition to their characteristic manifestations, acute hemolytic anemia with or without thrombocytopenic purpura. Acute hemolytic anemia is seen also as a complication of infectious equine anemia, a specific viral infection of horses ("swamp fever") (27). Recently a virus disease known as Kyasanur Forest disease (KFD) has been observed in South India in monkeys and humans as a cause of hemolytic anemia with marked erythrophagocytosis, leukopenia, and thrombocytopenia with or without purpura (28).

The demonstration by us of virus-red cell interactions in the circulating blood of patients with various types of cancer suggests an explanation for the anemia so frequently found in cancer (29). According to *Hallauer* and *Kronauer* (30) (1962) it is due to "a particular agglutinin" which they believe is a hemagglutinating virus as isolated by them in tissue cultures of human tumors. *Simpson* and *Belcher* (31) (1962), citing our work on the action of viruses on red cells, described a hemolytic anemia of rats produced by inoculation of a minute quantity of blood from rats carrying a transplantable rat tumor. In the same connection one can also cite the high incidence of hemolytic anemia documented in patients with Hodgkin's disease (32), leukemia (33) and lymphosarcoma (33a, 34).

The frequent appearance of Coombs'-positive hemolytic anemia and of antinuclear factors in the blood in SLE together with the occasional development of "hypersensitivity angiitis" have been cited often as supporting evidences for auto-immunity as the basis

of the disorder. Notwithstanding the attractiveness of this hypothesis one can draw up an equally persuasive argument in favor of regarding such auto-immune phenomena as the result rather than the cause of the disease. It would hardly be surprising if the large quantities of cytoplasmic and nuclear debris which flood the circulation in systemic lupus were to have the effect of a multiplicity of antigens. Indeed, antibodies have been shown to develop not only against nuclear DNA but also against nuclear histones and various cytoplasmic components (35). If it can be assumed that the cellular damage in SLE is produced by a necrotizing virus, the liberated cellular material, particularly nuclear material with which the virus combines energetically, may well be transformed into a highly active antigen.

The almost specific tropism of the causative agent of SLE for mesenchymal elements is manifested not only in severe destructive changes in the germinal centers of lymph nodes but also in many other derivatives of the mesenchyme including all blood forming elements. Surprisingly epithelial tissues are virtually untouched by the agent. This feature would appear to favor the concept of the LE agent as a mesenchymotropic virus rather than pantropic or epitheliotropic virus. On this basis it may be possible to regard auto-immune hemolytic anemia occurring in SLE as the result of antigenic modification of erythrocytes induced by their binding of the LE virus-like agent. If one accepts the premise that normal healthy donors are immune to this agent an explanation is then provided for the severe transfusion reactions occasionally encountered in SLE patients despite careful blood matching. As a consequence of this the natural antibodies in the donor serum may be expected to combine with the virus-conditioned red cells of these patients and thereby provoke hemolysis*. The same considerations apply to transfusion reactions encountered occasionally in patients with cancer in whom likewise no grouping incompatibility can be demonstrated.

Etiologic Significance of Virus-like Agents in Hodgkin's Disease and other Malignancies

A variety of microorganisms including diphtheroids and brucella have at one time or another held the spotlight as the cause of Hodgkin's disease. Lesions resembling Hodgkin's disease have been observed in patients on anticonvulsant drugs; in some instances these patients eventually developed the typical clinical picture of Hodgkin's disease (36). A transmissible lymphoma suggestive of Hodgkin's disease was created in mice by transplanting parental lymphocytes, which produced a "graft-versus-host" reaction and thereby incited an aberrant proliferative response of host lymphocytes (37).

The idea that Hodgkin's disease is caused by a virus has engaged the interest of students of the problem for many years. The chronicity of the disease and the granulomatous nature of its identifying lesions are compatible with the premise that the virus may be harbored in a localized manner for a considerable period of time ("slow virus").

Attempts to demonstrate a virus in cases of Hodgkin's disease have been far from conclusive. Macromolecular particles resembling virus have been reported in electron microscope studies. Lymph node triturates have been reported to cause edema or hemorrhagic changes in inoculated embryonated chicken eggs and to produce Hodgkin's-like

* Suggestion by *R. Shonk*, Middlesex General Hospital Laboratories.

changes in tissue cultures including large cytoplasmic inclusions (38). As in the case of diphtheroids isolated from the diseased tissue, it is difficult to prove that the virus-like agents were not pre-existent in the chick embryo or other living system employed for demonstrating a virus or were merely "passenger viruses" growing by preference in a diseased tissue (39).

The same considerations apply in connection with the search for causative viruses in human cancer (40). Although a known human virus species (Adenovirus Types 12 and 18) has been shown to produce tumors in hamsters when inoculated neonatally (41) and to enhance the growth in tissue culture of human embryonic cells with formation of giant and syncytial cells (42), there has been no indication thus far that these viruses are capable of inducing tumors in man. One may plausibly relate this lack of oncogenic potential of the adenoviruses for man to their high antigenicity for man as reflected in the high level of antibody against these viruses in human subjects. This circumstance presumably removes the adenoviruses from the category of "slow virus" infection as they concern man.

Thus far the causative role of viruses in human cancer is supported largely by a small number of epidemiological data. These include the higher incidence of leukemia and Hodgkin's disease in blood relatives and the occurrence of "nests" of leukemia, as in Niles, Illinois, where 8 children who attended the same parochial school developed leukemia within a few years (40).

It is apparent that one cannot apply *Koch*'s four postulates in attempting to evaluate the relation of viruses to cancer in man. These postulates require that 1. the etiologic agent should be found in every case of the disease but not in normal subjects with other diseases; 2. the agent should be cultivable in pure form; 3. the disease should be reproduced in animals inoculated with the pure culture; and 4. the agent should be re-isolated in pure culture from the experimental animals. It is not possible even with the best tissue culture techniques available today to isolate the responsible viral agent from *all* cases of a particular disease nor is it always possible to isolate such agents in pure culture. Although there is hope that epidemiological measures showing the increased frequency of a particular agent in focal or familial concentrations of human cancer may be applicable in some instances, protection by vaccination could require many years or even a generation to establish *(Bryan* 40). The concept of "slow virus" as the basic factor in cancer induction implies a deficiency of normal immune mechanisms of protection. Accordingly serologic studies for specific antibodies cannot be utilized in such an investigation.

Whether the present studies shed any light on the question of viral causation of cancer in man cannot be known without more evidence. The data obtained in the present studies are promising for what they indicate of the presence of a single antigenically homogeneous species of virus-like agent in the blood of patients with Hodgkin's disease, leukemias, lymphosarcomas, and various types of carcinoma. Of interest is the surprising similarity in the serological data obtained in the two series of studies despite the fact that the tests were separated by an interval of 12 years and performed by different persons. Although the relative proportion of positive results differed considerably in the two series the differences appear to be quantitative rather than qualitative, both series revealing a distinctly higher incidence of positive results in Hodgkin's disease than in the other malignancies and an extremely low incidence of positive results in control subjects.

The explanation for these quantitative differences is unclear. One may predicate that it lies in the difference in conditions under which the studies were made both in location and in individual techniques. Another factor may be the improvement in methods of treating Hodgkin's disease, particularly modern techniques of radiation therapy, which in some cases accomplish virtually complete cure. This possibility is further supported by the not infrequent detection of the "HD virus" in the initial tests of particular patients but not subsequently. The latter observation also has bearing on the question whether or not these blood-borne agents represent "passenger viruses" occurring secondarily in the tumor tissue and not primary incitants. In support of their *primary role* is our experience in the case of a patient with Hodgkin's disease in whom the first two biopsies of enlarged cervical lymph nodes failed to establish the diagnosis histologically. A preliminary diagnosis of "diffuse reticuloendothelial hyperplasia of probably reactive type" and "minor cellular atypism as in the presence of viral infection" was made without further comment although the suspicion of Hodgkin's disease was mentioned verbally to the patient's physician. Virus study yielded an abundance of virus type plaques in chick embryos and agglutination tests of the patient's red cells with HD antisera gave a positive result. Eventually the disease progressed and ended fatally in the form of advanced retroperitoneal Hodgkin's disease.

The reason for the distinctly higher rate of positive results in Hodgkin's disease compared with other malignancies is an arresting question. Conjecture favors the idea that the anergic state, which is common in Hodgkin's disease as in many known viral infections (e.g. measles), favors multiplication and dissemination of the virus. In other malignancies, less subject to the development of anergy, the virus may remain more securely hidden in the infected cell.

Much further work remains to be done before the claim can be justified that the virus-like agents represent a species of human polyoma virus, i.e. a virus capable of inducing a varied assortment of tumors in the same and different hosts. Multiple primary tumors are comparatively rare in the human although evidence exists that the incidence of leukemia and lymphosarcoma is higher in patients with carcinoma than in the ordinary population.

Of further interest is the fact that the SE polyoma virus of mice, hamsters and other animal species is known to be a hemagglutinating virus (43). The fortuitous circumstance that the virus-like agent uncovered in Hodgkin's disease and other human malignancies is also a hemagglutinating agent explains the ease with which it is detected in circulating blood despite the almost insuperable complexities of attempting to demonstrate its existence in tumor tissues.

Summary

The affinity of certain viruses for the red cell has been known for a quarter of a century, chiefly as an in vitro phenomenon useful in virologic diagnosis. The possibility has been suggested that the same phenomenon may play a role in the blood-borne transport of viruses within the living organism. That the phenomenon of viral adsorption to the red cell may also play a part in the pathogenesis of certain hemolytic syndromes has been reported by us previously. This aspect of viral infection may be the product of recirculation of red cells repeatedly through virus-infected tissues whereby they are enabled to accumulate a relatively large concentration of virus material upon their surface. For this reason the red cells in circulation may constitute a particularly satisfactory source of virus isolation in certain conditions.

Virus-like agents were isolated by us from blood samples of a large number of patients suffering from Hodgkin's disease, leukemias, lymphosarcomas, various types of carcinoma, systemic lupus erythematosus, infectious mononucleosis, and certain other conditions. Morphologically the plaques which developed in the chick chorioallantoic membrane were distinctive for each disorder and specific immune sera could be produced in rabbits with the allantoic fluid. When suspensions of the red cells of human patients suffering from each of these disorders were tested with the corresponding rabbit immune sera in serial dilution the presence of adsorbed viral antigen caused the cells to be agglutinated in accordance with a high order of specificity. Because of its simplicity this serological method has been found applicable in diagnosis in questionable cases of Hodgkin's disease and SLE. The affinity of these agents for the red cell in vivo may account for certain aberrant immunologic phenomena which are not uncommon in these disorders, including "auto-immune hemolytic anemia". The relatively sharp serologic specificities of the virus-like agents isolated in Hodgkin's disease and other malignancies and in systemic lupus erythematosus are compatible with the premise that these agents may be etiologically related to these diseases. As such they may belong in the category of "slow viruses" and remain latent for long periods of time unless provoked into activity by various extrinsic factors, e.g. sunlight or hydralazine in the case of SLE. The viruslike agent encountered in Hodgkin's disease and other malignancies bears certain similarities to the polyoma virus which can produce a wide variety of tumors in mice and other animals. The possibility of "vertical transmission" transplacentally before development of immunological competence of the embryo may account for the absence of immunity against such agents.

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EDITORIALS

Cellular Aspects on the Phylogeny of Immunity

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One way of studying the development of an organ system is to start the study in a phylum in which the actual organ system has reached the highest degree of organization or complexity, and then turn to less organized or less complex organs in other phyla. It is wise to keep in mind that there are no fossil records of most organ systems. This is certainly true of the lymphatic organs. The fishes deviated from our common ancestors about 300 million years ago, and modern fishes thus had a long time to develop fancy lymphatic organs if the selection pressure was such as to require this. We can thus expect to find a variety of "solutions" to the same basic problem. A study of the various "solutions" may also give some ideas towards what the "problem" was, in other words: Why lymphatic organs developed at all.

First Level and Second Level Lymphoid Organs

The last ten years of immunological research have given us good reason to divide the lymphoid organs into first level and second level. If a first level lymphoid organ is removed early enough in ontogeny development of certain parts of the second level lymphoid organs is reduced in the operated animal. First level lymphoid organs defined so far are the thymus and bursa Fabricii of chickens. The work of *Cooper, Peterson* and *Good* (1) has indicated that there is a dissociation of the immunological function in the chicken. The bursa of Fabricius controls the development of immunoglobulin production, and the thymus is largely responsible for the ontogeny of cellular immunity.

Thymus and bursa Fabricii are both lymphoepithelial organs in the sense that they represent an intimate relationship between epithelial or epithelial-derived cells and lymphocytes. Lymphocytes in both organs are characterized by rapid proliferation. It has been shown (2, 3) that thymus and bursal lymphocytes are at least partly derived from blood borne progenitor cells, which enter the epithelial primordia of these organs during histogenesis.