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Donald J. LaPorte

University of Nebraska Medical Center

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HETEROPHILE ANTIBODIES IN INFECTIOUS MONONUCLEOSIS

A REVIEW OF THE LITERATURE

Donald Joseph LaPorte

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College of Medicine, University of Nebraska

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Heterophile Antibodies in Infectious Mononucleosis

A Review of the Literature

I. Historical Introduction

A. Infectious Mononucleosis

"Drusenfieber" was the name given by Pfeiffer (1) in 1889 to the syndrome of enlarged cervical glands, fever, enlargement of the liver and spleen, occurring in children. Filatow (2) a Moscow pediatrician had discribed a similar condition in 1885, but had attached no name to it. Pfeiffer's terminology was translated by Williams (3) in 1897. In the third edition of Osler's textbook of medicine (4), published in 1897, this description was repeated.

In the following years the disease was frequently confused with leukemia, causing much surprise because all patients recovered.

In 1920 the disease entered its hematologic period. Sprunt and Evans (5) published a paper on "Mononuclear Leukocytosis in Relation to Acute Infections." They used the term "infectious mononucleosis" and emphasized the pathologic lymphoid cells characteristic of the disease. Longcope (6) in 1922 suggested that glandular fever and infectious mononucleosis were synonymous. Since then the concept has gradually been accepted among American writers.

B. Heterophile Antibodies

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Early in the history of immunology, the concept of strict specificity between antigen and antibody was challenged. Substances were found which, when injected into certain animals would elicit not only specific antibodies, but antibodies which reacted with antigens other than those involved in their production. In 1911, Forssman (7) published a paper in which he stated that "formation of sheep hemolysin results from the injection into rabbits of emulsions of guinea-pig organs (liver, kidney, adrenals, testes, brain, but not of blood). The hemolysins thus obtained are comparable in activity with those resulting from injection of sheep blood. Like the usual hemolysin, they consist of immune body and alexin. After one-half hour of heating at 56 degrees Centigrade, they are reactivated by rabbit serum, or still better by guinea pig serum. They combine with the same receptors in the sheep corpuscles as the usual sheep hemolysin. They are even more specific than the ordinary sheep hemolysin, for they do not dissolve ox corpuscles. ...These hemolysins are similar to normal hemolysin and both are different from that resulting from injection of blood." Forssman also stated that the formation of the "guinea-pig" sheep hemolysin occurs without any combination between the antigen and the hemolytic antibodies. This fact he used as an argument against the side chain explanation of immune body formation. Later workers (8,9) failed to substantiate this observation.

Taniguchi (10), in 1920 observed that when guinea-pig heart

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which contains Forssman antigen, was used as the antigen in the Wassermann test, there was considerable variation in the ability of the unknown sera to fix complement, depending on the amount of sheep cell hemolysin contained therein. This variable complement-fixing property could be modified by absorbing out this hemolysin with such Forssman antigen as guinea-pig kidney. Aware of the fact that normal sera might contain fairly large amounts of sheep cell hemolysin, he concluded that the substance constituted a source of fallacy in the Wassermann reaction when alcoholic extracts of heterogenetic antigen such as guinea-pig or horse heart were employed.

In 1924 Hanganutzui (11), reading the results of routine Wassermann reactions noted an incidence of strong agglutination of sheep red-cells. This serum, which proved to contain a high titer of sheep-cell antibodies, was found to belong to a patient injected therapeutically ten days before with horse serum. In this case, as well as in the other serum-treated individuals whom he subsequently studied, he demonstrated increased amounts of agglutinin against red cells of the horse, guinea-pig, and several other animals. These antibodies appeared about the tenth or eleventh day after injection and remained for a number of weeks; their presence was independent of the amount of serum administered or the number of injections. In the serum of no individual untreated with horse serum did the normally low titer of hemagglutinins approach the high values seen in serum-treated

individuals.

The essential features of these observations were confirmed two years later by Deicher (12). Indeed, in Europe the Paul-Bunnell test is often referred to as the Hanganutziu-Deicher reaction.

Paul and Bunnell (13) in 1932, attempted to establish a serologic test for acute rheumatic fever. Impressed by its clinical similarities to serum disease, in which Davidsohn (14) had found an increase of heterophile antibodies, they studied the heterophile antibody content of sera from cases of rheumatic fever as well as from a group of hospital patients suffering from other diseases. In the serum of one of this control group, a high titer of sheep cell agglutinins was demonstrated. This patient had infectious mononucleosis.

The following year, Bunnell (15) published the observation that a high titer of heterophile antibody occurs in most cases of infectious mononucleosis and that it was sufficiently characteristic to be useful as a dianostic aid. Subsequent clinical reports (16-30) emphasized the confirmative value of the sheep cell agglutination test in the diagnosis of infectious mononucleosis.

C. Discussion

Before further consideration is given to the value of the Paul-Bunnell test in the dighosis of infectious mononucleosis, it is necessary to review the circumstances in which the early investigators found themselves. Infectious mononucleosis was not

unanimously accepted as a definite disease-entity. The etiolgic

agent of this condition was unknown, and many authors considered

it as a nonspecific lymphocytic response to acute infection (31).

Furthermore, the clinical picture alone would not permit a diagnosis

with certainty because it was mimicked by so many other entities

(32-40). There may be no clinical signs or symptoms (32,35,37,

41). The hematologic features of infectious mononucleosis are

sufficiently characteristic in most instances to be of extreme

importance in helping to make the dianosis but by themselves they

are not specific (42-45) and were therefore useful only when

judged in conjunction with all other clinical findings. The

cardinal hematologic features include a relative and absolute

lymphocytosis plus the appearance of abnormal lymphocytes

(virocytes) (46) in the peripheral blood. Numerous observers

(35,46-52) have emphasized that the same features occur in

other diseases, chiefly of viral origin (acute virus hepatitis,

virus pneumonia, rubella, rubeola, roseola infantum, herpes zoster,

herpes simplex, influenza type B, upper respiratory infections,

undulant fever, rickettsial pox, allergic states). If there are

any differences between the blood picture in infectious mono-

nucleosis, on the one hand, and these other diseases, on the other,

they are differences in degree. The evidence of hepatic in-

volvement (53-58) was generally unknown at the time, hence could

not be used in arriving at the final diagnosis. It is obvious,

then, that evaluation of the specificity of this new test would tend to vary widely.

II. Heterophile Antibody Test of Paul and Bunnell: Technic (13)

The materials required to determine the titer of sheep cell agglutinins are the patient's serum, a suspension of sheep red cells, and physiological saline. (The determination of hemolysins requires complement in addition).

The serum is inactivated for fifteen minutes at 56 degrees Centigrade; if kept in the icebox its potency remains constant over a period of years (33). Starting at 1:4, dilutions are carried out as far as indicated. Sheep cells, collected weekly, are washed three times; from them a 0.67 percent suspension of packed cells is prepared. To each tube containing 0.5 cc of diluted serum, 1.5cc of the suspension of sheep cells is added. The tubes are shaken and are placed in a 37 degrees water bath for one hour, then kept overnight in the icebox. The following morning the tubes are gently inverted three times after which any tube in which there is microscopic agglutination of the red cells is considered positive.

III. Modifications of the Paul-Bunnell Test

A number of modifications of the original technic of Paul and Bunnell have been presented in the literature.

Stuart et al (59) recommend the omission of the one cc of saline which they say serves no useful purpose, and the use of

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a one percent suspension of sheep cells. They state that human

sera may contain antibodies which agglutinate sheep erythrocytes

in the cold and that this phenomenon is reversed by incubation

at 37 degrees Centigrade. Agglutination of sheep red cells by

infectious mononucleosis sera, on the other hand occurs at 37.5

degrees Centigrade. Agglutination of sheep red cells by in-

fectious mononucleosis sera, on the other hand occurs at 37.5

degrees Centigrade. This titer may be increased by overnight

refrigeration at 4 degrees Centigrade. In order to dispel any

possible "cold agglutinin" effect the test should be returned to

the water bath (37.5 C) for two hours and the final reading made.

Davidsohn (42) describes a more delicate test requiring

only two hour's incubation, and using smaller amounts of material.

He suggests the use of special narrow tubes, so that the end

point of the agglutinination may be more accurately detected

by the low power of the microscope. This method obviously gives

higher readings. It will be described in detail in a later

section.

Certain quick methods are also in use. Thus Butt and

Foord (26), in addition to the Paul-Bunnell technique, used a

microscopic agglutination technic by taking one loopful of the

patient's serum and four loopfuls of a two per cent suspension

of sheep cells in saline and making a hanging-drop preparation.

In cases of infectious mononucleosis and serum sickness almost

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immediate agglutination takes place.

Straus (60) presents two rapid methods. One, similar to the microscopic slide precipitation test as used in the Kline test for syphilis. He states that one may obtain prompt, more sensitive results than the rapid method of Butt and Foord (26) by this method. The second method, in which the centrifuge plays an important part, a series of serum dilutions in one cc. amounts is made. One cc. of a two per cent sheep cell suspension is added. Centrifuge for five minutes at about 2000 r.p.m. Shake and read.

Hollander (61) also uses the centrifuge. 0.5 c.c. of sheep cells is added to 0.5 c.c. of serum dilution and then centrifuged for five minutes at 1000 revolutions a minute. Hollander makes no reference to Straus.

Rappaport and Skariton (62) and Maloney and Malzone (63) in 1949 each devised a screening test for infectious mononucleosis. Maloney and Malzone describe their method as given below:

"On a glass slide at room temperature 0.1 cc. defibrinated sheep blood was mixed with 0.2 cc. serum to be tested. Results were positive only if 3 or 4 plus macroscopic clumping occurred within 30-60 seconds. The heterophil antibody test was carried out on the same serums, using the Paul-Bunnell method, and a serum dilution of 1:128 was considered the lowest positive level. Sheep cells were preferably used fresh, but defibrinated sheep blood kept at 5 C. for two week gave reliable results.

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Inactivation of serum was unnecessary. Serums stored in the ice-box lost potency slowly, but if kept in the deep freeze the heterophile antibody was well preserved for long periods. The heterophile antibody in infectious mononucleosis is active at 37 C. as well as at lower temperatures."

The rapid slide test can give positive results with cold agglutinins (which may be abolished by warming to 37 C.) or Forssman antibodies (which may be absorbed by guinea-pig kidney). There was no evidence that blocking, incomplete or hyperimmune heterophile antibodies occur in infectious mononucleosis. Eyguem and Polliachock (64) in 1951 were unable to demonstrate incomplete antibodies in sera of patients with infectious mono-nucleosis.

Vaugn (65) used the method of Maloney and Malzone, but modified it in that suspension of cells in saline was used rather than a 2:1 ratio of serum to undiluted sheep-cells. In his series of 16 sera giving positive Paul-Bunnell tests, the slide test was also positive. In 252 normal sera giving a neg-ative Paul-Bunnell test, the slide test was also negative. Tannen (66) recently published details of a similar screen-ing procedure.

Results of modifications of the Paul-Bunnell test which employ the centrifuge must be evaluated in the light of the knowledge that this method tends to yield higher titers than the sediment technic (67).

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IV. Specificity of Test

It was known before Paul and Bunnell published their find-
ings in 1932, that the sera of normal individuals contained
varying amounts of heterophile antibody (68 - 69). This was
shown to be elevated in individuals who had received injections
of horse serum (12,14,17,27,70 - 72). The reaction, therefore,
was considered to be non-specific and it was only on an empirical
basis that a diagnostic titer could be established. It is not
surprising that throughout the literature there is considerable
variation in the lowest titer considered diagnostic. It has been
variously determined as 1:8 (73), 1:32 (13,15,21,33,74,75), 1:56
(21,42,76) 1:64 (77,78), 1:320 (59), and 1:512 (79). This will
be commented on later. If one judges the extent of the test's
specificity by the percentages of positive serologic tests in
twenty-one reported series totalling 1,643 cases taken from the
literature, the extremes range from 50 to 100 per cent, with the
overall average of approximately 83 per cent (26,33,35 -37,40,
55,56,75,78,80 - 90). Paul and Bunnell, in their original
article, did not consider their test to be specific for infectious
mononucleosis. In their series of controls one of the patients
suffering from an obscure disease, never diagnosed, and terminating
fatally gave a positive titer of 1:640. The following comment
was made by Paul and Bunnell (13) in this article:

"...In spite of the fact that the limits of the reaction

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which we have described have not been tested, it would seem to
be of diagnostic value. In a sense, however, it seems to be of
more theoretical than practical interest. Theoretical interest
centers about the fact that heterophile antibodies may be
produced or enhanced during the course of human infectious
diseases,' and in one disease in particular, in which the etiology
has not been established. Furthermore, that two clinical entities
with widely differing symptomatology such as serum sickness and
infectious mononucleosis, would elicit the same type of serologic
response is also worthy of interest. ... On the other hand it
is also conceivable that the phenomenon which we have described
is in the nature of an isoagglutinin response to the presence
of an excess of abnormal cells either present in the blood or
elsewhere."

V. Seronegative Cases of Infectious Mononucleosis

It has been stated that prior to the advent of serologic
confirmation of the diagnosis of infectious mononucleosis,
clinical and hematological manifestations were recognized,
neither of which was specific for the disease, but which, in
conbination, were confirmatory, and as such, constituted,there
criteria by which specificity of the heterophile agglutination
test was determined. As early as 1935 (43), it was stated that
a negative heterophile antibody test does not rule out in-
fectious mononucleosis, even if the test is repeatedly negative.

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This statement was affirmed by many authors (33,35,42,75,85, 91 - 95). Rosenthal (16) in 1933 concluded that if the clinical picture is sufficiently characterestic, a negative Paul-Bunnell test does not preclude or diagnosis of infectious mononucleosis anymore than a negative Wassermann reaction rules out syphilis. The inherent conclusion is that the heterophile antibody absorption test is not 100 per cent specific, and that the existance of a seronegative form of the disease is necessary. Davidohn (42) in 1937 stated definitely that there were two forms of infectious mononucleosis-seropositive and seronegative, and considered it desirable to separate them. Kaufman (85) does not agree with Davidsohn that it is necessary or desirable to separate seropositive from seronegative cases of what is considered to be the same disease. Goldthwait and Eliot (94) in 1951, reported a mild clinical and subclinical epidemic most probably of infectious mononucleosis in a detachment of men sent to the arctic. All twenty six men showed abnormal mononuclear cells in their blood, while only five developed symptoms sufficient to classify them as clinical cases, and only one had a heterophile agglutination together with liver damage. While emphasizing the fact that "one cannot be dogmatic in defining criteria for diagnosis of the disease process "infectious mononucleosis" when the etiologic agent is unknown and the agent-host relationships are not understood, "they concluded that "an entity exists called in our present ignorance "subclinical

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infectious mononucleosis' ". Bender (95) in 1952 remarked at
the "extreme rarity of seronegative cases of infectious mono-
nucleosis in the age group seventeen to thirty-two years".

VI. Sources of Error

The existence of seronegative cases of infectious mono-
nucleosis is a necessary corollary to the lack of complete
specificity of the heterophile-agglutination test. An over-
all average of 83 per cent specificity has been previously noted
in a total of 1,643 reported cases. However, these statistics
are open to question for several reasons: Interpretation of a
positive test has varied widely in different reports because of
the several technics employed, and has varied even when the
technics employed have been similar. Barrett (96) obtained a
frequency distribution curve of agglutinin titers in normal in-
dividuals significantly different from that obtained by Stuart
et al (59) using the same technic. This type of descrpancy may
be accounted for by differences in technics recording dilutions,
true factors, end points, and by the frequency of sampling.
Definitions of a normal titer vary. It is also confusing that
not all titers are expressed as the true dilution, and is, the
final dilution; for example, Paul and Bunnell (13) call tube one
1:4 whereas it is really a 1:16 dilution of serum. Davidsohn
(42) designates his first tube as 1:7; Bernstein (74) desingnates
his first tube as 1:20. Another factor affecting the above

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mentioned statistics is that most workers have not attempted to confirm their agglutinations with absorption test (to be described). Again, when the specificity of a serologic test is still under investigation, the basic dianoses in the cases reported should be beyond question; but this is not possible because no specific clinical or laboratory yardstick is available. Then again, if the investigator rigidly requires tha all suspected cases yield positive serologic evidence before a diagnosis of infectious mononucleosis can be made, it follows that 100 per cent specificity will necessarily be obtained in any study such as that of Van Ravenswaay (21).

In assuming the existence of a seronegative type of infectious mononucleosis, more is presumed than statistical evidence of less than 100 per cent specificity for the heterophile agglutination test. The possibility that a positive titer may have occurred in the disease at a time other than that at which the patient's serum was taken must also be ruled out. Hence grounds on which "seronegative" cases are reported are open to criticism. Four factors contribute to this: 1) Failure to perform agglutination tests early enough in the illness; 2) Failure to perform repeated agglutination tests late enough in the illness; 3) Failure to perform confirmatory absorption tests on serum with low titers and 4) failure to exclude cases simulating but not infectious mononucleosis. As Bender (95) states, "if cases

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are classified as serologically negative on the basis of a single test during the first week of the illness, the results are understandable." There are sources of error inherent in the agglutination phenomenon itself. Keiper (97) points out that the agglutinability of erythrocytes from different sheep varies widely and cells from an occasional sheep react only slightly with the serum of known cases of infectious mononucleosis. Lesser variations in agglutinability are encountered in cells drawn periodically from the same sheep and there is some variation following storage of cells. That the agglutination titer is influenced by the age of cells was known as early as 1934 (98, 21). Variations in agglutinin titers may be obtained by varying the concentration of cells used in the test,by varying the temperature at which the cells are incubated and by varying the length of incubation (59).

VII. Nature of Sheep Cell Agglutinins

In 1911, Forssman (7) first observed that when watery suspensions of the organs of a guinea-pig were injected into rabbits, a hemolysin for sheep erythrocytes was produced. This is referred to as a Forssman antibody. It reacts with an antigen derived from a species of animal not closely related to that which provoked its production.

Forssman antigen, in addition to being present in guinea-pig tissues, is found in many warm-and cold-blooded animals,

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including the horse, cat, dog, mouse, tortoise, etc. These are
referred to as animals of the guinea-pig group, while those
animals from which the antigen is absent are of the rabbit group.
Examples of these are the man, rabbit, ox, pig, rat, toad, etc.
The distribution of this antigen throughout nature seems to be
in a haphzard fashion, although evidence has been recently pre-
sented that the antigens occur, in animals, at least, in an
orderly manner (99). It appears that when Forssman antigen
is present in the tissues it is absent from the red cells of
that species, and vice-versa. These points help toward the
understanding of the various absorption tests carried out by
different observers.

Paul and Bunnell (13) at the time of their publication in
1932, assumed that the heterophile antibodies in infectious
mononucleosis were Forssman in character. There was no reason
to suspect that the titer of sheep cell agglutinins seen in
infectious mononucleosis was other than an increase in the
normal heterophile antibody content of human serum, nor was
there any indication that these antibodies differed in any
respect from those seen in serum sickness. Very soon, however,
there appeared in the literature data incompatible with this
conception. It was noted that sheep-cell agglutinins appeared
equally regularly in patients with infectious mononucleosis
irrespective of the blood group to which they belong (74,100,101).

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This would be a paradoxical phenomenon since human group A cells contain Forssman antigen (102). Should Forssman antigen and antibody occur together some sort of reaction would be anticipated (99). Furthermore, the infectious mononucleosis antibodies are poorly absorbed by guinea-pig tissues which efficiently remove Forssman antibodies (59,103).

Continued investigations by Stewart and his corroborators (104)(105) cast doubt on the Forssman character of the anti-bodies found in individuals treated with horse serum, since the antibodies are removed to a considerable extent by non-Forssman containing rabbit red cells. At this point Bailey and Raffel (106) found that the antibodies in the serum of infectious mono-nucleosis cases could be absorbed by boiled or autoclaved ox red cells in contrast to the antibodies in normal serum. They were thus not Forssman in type. This finding was promptly confirmed (107) and extended by Stuart et al (108 - 110) to show that in infectious mononucleosis there may be an increase of antibodies against red cells of sheep, goat, horse, or ox, bu not rabbit, dog, or guinea-pig; whereas after injection of horse serum there is increased titer against the cells of all of these species (111).

Davidsohn had been working on the nature of the heterophile antibodies in infectious mononucleosis similtaneously with the workers just mentioned (42). His results were more or less in accord with Stuart. Thus it would seem that there are at least

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three types of sheep cell agglutinins-one present in normal serum,
absorbed by guinea pig kidney and not by ox cells; another in
the serum of individuals treated with horse serum, absorbed by
both guinea-pig kidney and ox cells; and still another in the
serum of patients with infectious mononucleosis, absorbed by ox
cells and not guinea-pig kidney.

Further support for the differentitiation between the types
of antibody response seen in infectious mononucleosis and
serum disease was provided in 1938 by Davidsohn (112) who ob-
served higher titers of isoagglutinins in the latter, but normal
values in the former.

Stuart et al (108), as a result of inhibition and adsorption
experiments, found that the sheep cell antibodies in infectious
mononucleosis are not Forssman in type, because they reacted
with alcohol-treated sheep and beef cells, but not with the
alcohol extracts of the cells.

To understand this conclusion, it should be noted that
Forssman antigen is a protein-lipoid complex (99). The lipoid
component, which acts as a hapten, can be extracted with alcohol
and reacts with the anti-serum.

Sohier (113) in 1939 presented evidence that the titer of
the normal Forssman sheep cell agglutinins may increase before
the development of the typical heterophile antibody of infectious
mononucleosis.

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VIII. Wassermann Reaction in Infectious Mononucleosis

Hanganutziu (35) made note of the incidence of strong agglutination of sheep red cells in the serum of a patient injected with horse serum. It is significant that this observation was made while he was doing a routine Wassermann test.

Taniguchi (114) made mention of the fact that use of guinea-pig or horse heart, which contain Forssman antigen, in the Wassermann reaction constituted a source of fallacy. Glanzmann and Ottensooser (20), in 1935 noted that, if in the course of performing a routine Wassermann test agglutinations of sheep cells is noted, one may diagnose an otherwise unsuspected case of infectious mononucleosis. This false positive Wassermann reaction in infectious mononucleosis lasts only a short time, but many writers have drawn attention to it (115 - 120). The reason for its occurrence is not understood. Sprunt (121) in 1933, suggested that it might in some way be related to the presence of the heterophile antibodies in the serum. Hatz (117) in 1938 described a case of infectious mononucleosis of a febrile type in which there was a positive Paul-Bunnell test and a positive Wassermann reaction, associated with a negative Kline test. As the patient recovered, both the Paul-Bunnell test and the Wassermann reaction became negative. Hatz concluded that this "seems to point to a similarity in the mode of formation of the antibodies concerned". Bernstein (120) indicates that the

apparent relationship between sheep-cell antibodies and occasion-
ally occuring false-positive Wassermann reactions is not so.
He offers three facts in substantiation of this statement: 1)
partial removal of sheep cell agglutinins makes the complement-
fixation test more positive rather than decreases its strength
(so that any binding effect of these agglutinins upon the sheep
red blood cells to prevent hemolysis is inconsequential); 2)
a positive Wassermann reaction may occur in the presence of low
titers of sheep-cell antibodies while a negative Wassermann
may accompany extremely high titer; 3) sheep cell antibodies
may persist for many months after the Wassermann test has become
negative and may even increase in titer coincident with a re-
version of the Wassermann to negative. It has since been pointed
out that these antibodies can be absorbed without interfering
with the positivity of the Wassermann test (117,120).

The incidence of false-positive Wassermann reactions in
cases of infectious mononucleosis has been variously reported
as 3.6 per cent (122), 10 per cent (119) 13 per cent (118)
and 18 per cent (120). Tidy (32) noted a transient false positive
Wassermann reaction in 50 per cent of cases seen in the London
epidemic of 1930, and Gooding (116) reported a positive reaction
in 59 per cent of 27 cases from that epidemic. Davis (123)
considers as unreliable all reports prior to 1930 because ade-
quate standardization had not yet become widespread. He gave as

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a diagnostic criterion a repeated positive reaction to more than one kind of test, or to the same test in two different laboratories, that becomes negative after a few weeks or months without antisyphilitic therapy.

False positive Wassermann reactions ordinarily appear during the second week of the disease although they can appear earlier (118). Sadusk (118) emphasized the importance of frequent testing as against a single examinations. In his series the incidence rose from 8 to 13 per cent when tests were repeated at regular intervals.

The Wassermann reaction is usually weak (123) and reverts to negative within two weeks, although it may persist as long as three months (118).

IX. Differential Absorption Test

It has been pointed out in the last section how various experiments led to the conclusion that there are three types of sheep-cell agglutinins—one present in normal serum absorbed by guinea-pig kidney and not by ox cells; another in the serum of individuals treated with horse serum, absorbed by both guinea-pig and ox cells; and still another in the serum of patients with infectious mononucleosis, absorbed by ox cells and not guinea-pig kidney. It remained for someone to convert these observations into a practical laboratory procedure for the differentiation of these types of agglutinins. Davidsohn, (42)

in 1937 published the details of a differential test which utiliz-
ed absorption with boiled beef antigen and boiled guinea-pig
kidney. He also gave in detail the only major modification of
the original Paul-Bunnell test to appear in the literature since
1932. This modification makes use of smaller amounts of material
and requires only two hour's incubation. For the details of
his report the reader is referred to his article.

Davidsohn (42) presented a serologic study of thirty cases
of infectious mononucleosis, of seventeen cases of serum disease,
of a group of borderline cases indistinguishable from infectious
mononucleosis and horse serum injection. The titers of the
sheep agglutinins were determined before and after the absorp-
tions and were estimated interms of the final dilutions. The
effect of the absorptions was expressed in the percentages of the
antibodies that were removed.

Davidsohn (42) emphasizes the different behavior of the
sheep agglutinins in the serum of normal persons and of persons
with serum disease and with infectious mononucleosis with re-
gard to the different antigens as shown by the results of his
differential test. He states the principles of his absorption
test as follows:

"The failure of the guinea-pig kidney to remove the agglut-
inins for sheep erythrocytes from the serum of patients with
infectious mononucleosis establishes that the heterophilic

antibodies in that disease are not of the Forssman type. The
readiness with which beef erythrocytes removed the antibodies
in serum disease is contrasted with their failure to do it in
normal serum. It is apparent that absorption with beef erythro-
cytes cannot be employed for the separation of infectious mono-
nucleosis from serum disease but that absorption with guinea-
pig kidney can clearly differentiate the two conditions. Re-
moval of the agglutinins for erythrocytes of sheep with beef
erythrocytes and the failure of the guinea-pig kidney to remove
them completely establishes the diagnosis of infectious mono-
nucleosis, while removal of the sheep agglutinins with the
guinea-pig kidney excludes mononucleosis."

Striking evidence of the value of the differential test
was evidenced in those cases where the clinical and hematologic
picture suggested infectious mononucleosis and the titer of
sheep cell agglutinins was borderline (1:56 and 1:112).

Davidsohn (42) concluded that the differential test for
infectious mononucleosis "is of deciding diagnostic value (a)
for the exclusion of cases that are clinically and hematologi-
cally indistinguishable from infectious mononucleosis and that
have a so-called borderline titer of heterophile antibodies –
1:56 or 1:112; (b) for the recognition of late cases of infec-
tious mononucleosis with a relatively low titer of heterophilic
antibodies, and (c) for the recognition of cases that are com-
plicated by a recent therapeutic injection of horse immune serum
or by serum disease."

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X. Modifications of Test

The differential test as Davidsohn (42) described it has gone through little change to the present time. In this country, there are two heterophile antibody tests commonly in use; that described by Paul and Bunnell originally with various modifications as noted, and Davidsohn's method; which in itself a major modification of the Paul and Bunnell technic. Kaufman (85) in 1944 published minor changes which he advocated in the performance of the sheep cell agglutination. The method he presents in his article is that used by Annis Thompson (124) who had performed over two thousand tests by the Davidsohn technic and had used the modifications since 1939. The changes are described as follows:

1.) Instead of inactivating the serum of thirty minutes at 56 degrees Centigrade, it was inactivated for four minutes at 61 degrees Centigrade.

2.) Instead of reading results of the agglutination after incubating for two hours at room temperature and again after overnight in the icebox, the final results were read after centrifuging at high speed for five minutes, then shaking thoroughly with the fingers.

3.) Instead of absorbing sera for one hour, five minutes, were found to be satisfactory if the tubes were shaken thoroughly during that time. This applies to both the guinea-pig kidney and beef erythrocytes.

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4.) The expression one plus, two plus, and three plus are not used; merely positive and negative.

5.) All results were read macroscopically because after centrifuga ion there was not enough diagnositic difference to warrant the use of the microscope.

A carefully controlled series of tests showed that the two methods give eseentially similiar results. This modification is believed to be more desirable in that much time is saved.- more tests can be done per day, and the speed of diagnosis is increased significantly.

XI. Normal Values

Prior to the advent of Paul and Bunnell, it had been known for some time that normal sera may contain agglutinins and hemolysins against sheep erythrocytes, but only in low titer. In the one-hundred controls used by Hanganutziu (11), only four yielded hemagglutinins and only in low titer.

In Davidsohn's (68) investigations into horse-serum sickness, he found that in 450 cases used as controls only 4.2 per cent showed the presence of agglutinins for sheep blood, and then only in the strongest dilution (1:4). Later (71) in another group of 850 normal cases, he found 9 per cent had agglutinins of 1:4, a few of 1:8, and none in higher dilutions.

Paul and Bunnell (13) in their original paper found a low titer of antibodies in 275 controls, with one exception,

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an obscure case, never definitely diagnosed.

Weinstein and Fitz-Hugh (23) in 1935, collectted from the literature 2300 "controls", consisting of normal persons and patients with diseases other than infectious mononucleosis and serum sickness. Of these 37 per cent had sheep-cell agglutinin titers of 1:32 or less, and none exceeded 1:128.

Davidsohn (71) studied sera from 217 normal humans and found 99 per cent of titers to be 1:28 or less, and none more than 1:56. The antibody of normal serum was absorbable by guinea-pig kidney (i.e., true Forssman type of antibody), but showed only nonspecific absorption with beef erythrocytes.

Stewart and associates (59), employing 0.5 per cent sheep-cell suspension and incubation for two hours at 37.5 degrees Centigrade examined the serum from 300 normal persons or patients with diseases other than infectious mononucleosis. They found a titer of 1:80 or less in 99 per cent of their tests; 1:160 in 0.7 per cent and 1:320 in 0.3 per cent.

Bunnell (15) examined 1600 sera from the Wassermann lab-oratory. When his results are converted into terms of final dilution, the titer was 1:64 or lower in 99.2 per cent of the specimens tested. A titer of 1:128 occurred in the remaining 0.8 per cent.

Butt and Foord (26) employed the same technic as Bunnell in examining the serum of 412 adult hospital patients. When

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their results are converted into terms of final dilution, the highest titer obtained was 1:64.

Bernstein (33) found a maximum titer of 1:80 in 300 hospital patients. He used a method similiar to that originally describ-ed by Paul and Bunnell (13).

Barrett (96) examined the serum of 100 apparently healthy people, employing 0.5 per cent sheep cell suspension and incu-bating the tubes at room temperature prior to reading. The maximum titer was 1:20.

Smeall (125), employing the technic of Paul and Bunnell, examined the serum of 765 patients not suffering from infectious mononucleosis or serum sickness. The highest titer obtained was 1:64.

Leibowitz (126), examined the serum of 200 normal/adults for heterophile antibodies and found no titer higher than 1:56. The titer is expressed as the final titer after the addition of all diluents. He used the technic of Davidsohn (127).

In summary, a titer of 1:56 may be considered the upper limit of normal in apparently healthy adult persons when ex-amined by the technic of Davidsohn wherein the tubes are in-cubated at room temperature for two hours prior to reading. With the variations in technics indicated and with the varying methods of selecting "normal" persons for study, the upper limit of antibody titer has been found to be between 1:20 and 1:320.

Females seem to have a slightly higher average titer than males
(128).

XII. Range of Titer in Infectious Mononucleosis

A. Time of Appearance

Bernstein (33) states that almost without exception the
Paul Bunnell test is positive, if it is going to become so at
all, when first performed, for it is usually not until four or
five days have elapsed that the correct nature of the illness
is suspected. In a case in which sheep-cell antibodies are
not elevated Bernstein recommends that the test be repeated
at regular intervals for a month after the onset of the illness
before efforts to obtain serologic confirmation of the diagnosis
may be relaxed. Hoagland (129) confirms this observation.

The Paul Bunnell test may antedate glandular enlargement
or any of the other clinical features (33). Sheep cell
agglutinins may be increased several days before any distinct
abnormalities are recognized in the leukocytes. In one case
(74) with a long prodromal period, an elevated titer was observed
twelve day's before the abnormal count was established.

Kaufman (85) in 1944 concluded that the heterophile anti-
body reaction may become positive as early as the third day,
but occasionally not until the second month or "not at all."
He confirms, however, the observation by Lyght (29) that "the
diagnosis (of infectious mononucleosis) can usually be arrived

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at in advance of the aid promised by the agglutination procedure.
If sheep cell agglutination tests are readily available, of
course, they may be applied as clinching evidence".

These authors (85,29) apparently relegate the serologic
test for heterophile antibodies to a confirmatory status. As
late as 1952 Bender (95), while expressing his confidence in
the sheep-cell agglutination test as a diagnostic aid, stated
that all patients with infectious mononucleosis characterized
by a positive blood smear have shown a diagnostic titer of
heterophile antibodies, provided the blood was tested serially
through the second week of the disease. (146 hospital and 194
ambulatory cases were included in his series). It was his
belief that the reporting of so-called "seronegative" cases   .
was done after random testing of serum, and he concludes that if
the test is not positive by the beginning of the second week,
"other leads should be pursued". He was aware, however, of
instances in which the heterophile antibodies appeared late in
the disease (32).

In Sturgis' textbook of hematology (130) published in 1948
it is stated that heterophile antibodies usually appear early,
i.e., within the first two or three weeks of illness.

Hoagland (129) in a series of fifty-six patients noted that
in the first week of the illness 95.3 per cent became positive,
and stated that if the test was not positive by the fourteenth
day of illness, it was very infrequently positive thereafter.

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He used absorption studies and considered as positive a titer of
1:56 in a non-absorbed serum and 1:28 in a guinea-pig kidney
absorbed serum.

Kaufman (85) reported one positive agglutination test on
the third day of illness, one on the fourth and fifth day and
many on the sixth. Werlin, Dolgopol and Stern (83) report a
positive reaction on the second day of illness. Bernstein (120)
and McAlpin (131) report a positive reaction on the third day.
Friedman and Beer (73) reported a positive reaction on the fourth
day in one patient, as did Worms and Demanche (132). Bunnell
(18) reported a positive reaction on the fourth day in two patients.
Many other reports confirm that significantly positive sheep
cell agglutinins are usually attained withing the first two or
three weeks of illness (55,75,86,87,90).

This is not always the case. In a series of 24 patients
Leibowitz and Brody (57) noted that significant elevations first
occured on the thirty-third day in one case and on the thirty-
first day in another case. Abrams (133) reported a case of
infectious mononucleosis with intense jaundice of unusual dur-
ation (at least eleven weeks) in which the antibody titer was
negantive on the fifth week, 1:28 at seven and one-half week,
1:112 at eight and one-half weeks, finally reaching a titer of
1:896 in the tenth week of illness. Himsworth (134) reported
one case in which the antibody titer did not develop until

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ten weeks after the onset of the disease. Bakst and Leibowitz (135) in 1952 report a case in which the diagnosis of infectious mononucleosis, suspected from the onset, could not be made until the seventh week after the onset of illness when for the first time both serologic and hematologic tests became "positive". Other exceptional instances in which the agglutinins have not been detected until the second and third months after the onset of illness are found in the literature (74,85,87,132,136).

B. Duration of Antibody Response

Obviously an important factor determining the duration of increased titers of antibodies is the height of the level attained. The duration varies within wide limits. Bernstein (33) states that an elevated titer may disappear as rapidly as two weeks after its appearance. In one of his cases, the earliest return to a normal level occurred withing seven weeks of the onset of illness (from 1:1280 to 1:80) but usually the interval was four or five months. In two cases increased titers existed five or six months later. Bernstein concludes that in such cases infectious mononucleosis may be diagnosed in retrospect long after clinical recovery.

Davidsohn (42) observed abnormal titers in ten cases from 26 to 114 days after the onset of illness, with an average of 56 days. Goldthwait and Eliot (94) noted the heterophile titer reached normal after 56 to 296 days—an average of 119 days.

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They make note of the fact that clinical recovery is most rapid, and that serologic recovery is slower than hematologic return to normal.

Kaufman (85) concluded from his series of seventy-eight cases that a positive heterophile reaction usually remains so for two to four months, and notes that it may remain positive for nine to twelve months. Leibowitz (57), Kaufman (85) and Sturgis (130) agree that "so-called" infectious mononucleosis antibodies rarely persist in the serum beyond six months after their initial appearance. Hoagland (129) reported his latest recorded positive reaction between the seventy-second and ninety-third day. His observations were in general agreement with those of Bernstein (33) and Kaufman (85) in that the duration of a positive heterophile reaction was most variable, persisting usually for at least three weeks and often for over one month. The earliest fall from a diagnostic to a subcritical titer occurred between the seventh and tenth days of illness. In one instance the titer changed between the eight and twenty-first and in another between the tenth and thirty-second days.

C. Peak of Titer

It has been noted that an important factor determining the duration of increased titers of antibodies is the height of the level attained. Kaufman (85) believes that, as a rule, a positive test will be found for a longer period of time if the

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reaction was at the same time positive in high dilution. However Bernstein (33) states that the titer of sheep cell agglutinins bears no relation to the severity of the disease or to the degree of lymphocytosis. He states that the usual titer ranges between 1:320 and 1:10,240. Values as high as 1:163,840 (137) and 1:81,920 (77) have been occasionally recorded. In general, the titers of agglutinins and hemolysins are closely comparable (110); rarely the hemolysins are distinctly lower (138). Southam (139) states that "early work on heterophile reactions used a hemolytic system; comparison with agglutination titers is impossible".

Leibowitz (140) makes note of the fact that many cases of infectious mononucleosis never attain very high titers. In his study of twenty-five cases, three had maximum titers of 1:448, five cases had maximum titers of 1:896, and the remaining seventeen showed maximum titers of 1:1792 and above. He emphasizes that approximately one-third of this series of cases required absorption tests for diagnosis at all times during observed periods of their illness.

We have seen the results of the heterophile antibody test when this particular technic is applied to the serum of normal persons. We have also noted how the specificity of the test was increased by the differential absorption test of Davidsohn. We must now determine the sensitivity of the test; i.e., de-

termine what are the results when this same technic is applied
to the serum of patients with illnesses other than infectious
mononucleosis.

XIII. Heterophile Antibody in Other Conditions

A. Serum Therapy

Before Paul and Bunnell evaluated their heterophile antibody
test for infectious mononucleosis, it was a well-known fact
that serum sickness gave increased titers of heterophile anti-
bodies. It was due to the similarities between serum sickness
and active rheumatic fever that Paul and Bunnell, in their quest
for a serologic test for rheumatic fever, accidentally discovered
the heterophile antibody increase in a patient with infectious
mononucleosis.

Hanganutziu (11) in 1924, while performing a routine
Wassermann test first noted high titers of heterophile antibody
in the serum of patients who had received therapeutic injections
of horse serum. As has been noted (page 3), the agglutinins
appeared ten or eleven days after injection and remained for
several weeks, and Hanganutziu's observations were confirmed by
Deicher (12) two years later.

Minkenhof (27) in 1935, and Beer (111) in 1936 observed
that an injection of horse serum into a patient with infectious
mononucleosis does not produce any further increase in sheep-
cell antibody titer beyond that which already exists. Serum

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sickness may appear but the administration of horse serum is fraught with no more danger than in a normal individual (72, 111,141). Likewise, skin tests with horse serum do not necessarily indicate any abnormal sensitivity (111).

Davidsohn (14,70,71) noted that the increase of heterophile antibody is especially high if the injection is followed by serum disease. The titers easily approached those found for infectious mononucleosis (69).

As has been seen (page 18) the heterophile antibodies in serum sickness differ from both the Forrsman antibodies in normal serum, and the heterophile antibodies in infectious mononucleosis, and are clearly differentiated in the differential absorption test of Davidsohn.

B. Infectious Diseases

By 1940, when Bernstein (33) made his survey of the literature, it was stated that the Paul-Bunnell reaction has been found positive only rarely in diseases other than infectious mononucleosis. Many thousands of tests had been carried out in a host of clinical conditions with negative results. These include the common infectious diseases, the exanthemata, a variety of hematological disorders, syphilis and the other diseases diagnosable by agglutination tests of one sort or another; such maladies associated at times with false-positive Wassermann reactions as yaws and rat-bite fever, and many others having any feature in

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common with infectious mononucleosis (74,120). Indications that an increased heterophile antibody titer may be found in infectious disease had been presented in the literature, however, as early as 1928, when Bailey (166) showed that rabbits infected with Bacillus leptisepticum produced heterophile antibodies. Bailey and Shorb (167) later (1931) found that rabbits infected with pneumocci produced demonstrable heterophile antibody titers. In 1935, Buchbinder (99) listed an impressive number of bacteria themselves containing Forssman antigen.

In 1936, Rockwell and VanKirk (168) summarized an article dealing with heterophile antigen production by bacteria and plants as follows:

1.) B. mucosus is a high produder of heterophile antigen both exogenous and endogenous.

2.) N. meningitidis and H. pertussis contain moderate amounts of heterophile antigen.

3.) Freshly isolated strains of H. influenza contain some heterophile antigen.

4.) Several strains of B. abortis, N. gonorrhoeae and B. acnei did not contain any heterophile antigen.

5.) Some evidence is produced that heterophile antigen may be capsular as well as somatic in origin.

Finally, Bornstein (169) in 1942, reported an increased heterophile antibody titer in a patient with an Esherichia coli

infection.

An occasional increased titer had been reported in scarlet fever, rubeola, tuberculosis, and filariasis (142). Young, Storey, and Redmond (143) in 1943 reported increased titers of sheep cell agglutinins found in the serum of patients with primary atypical pneumonia. Eaton, Murphy and Hanford (144) in the following year found now titers above 1:80 in fifty-six patients with atypical pneumonia. When account is taken of the equal volume of sheep cells which these workers added to the serum dilutions, these titers as reported should be doubled. In 1950, Bordone (145) noted a positive Paul-Bunnell reaction in a case of epidemic parotitis.

Soon after Bernstein's review of the literature, there began to appear numerous reports of sheep cell agglutinins found in the serum of patients with infectious hepatitis (48,57,144,146). Eaton, Murphy, and Hanford (146) described a maximum titer of 1:160 (becoming 1:320 when recalculated on the basis of final dilution) in nine per cent of two hundred twenty-two serum specimans from cases of viral hepatitis. Barker, Capps and Allen (47) Dempster (147), and de Vries (142) reported that a heterophile antibody titer was absent or negative in infectious hepatitis, but gave no data. It is presently agreed that in a certain number of cases of infectious hepatitis, a significantly high titer of sheep-cell agglutinins can be found. Cohn and

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Lidman (53), in 1946, impressed by the similarity of symptoms
of infectious hepatitis and infectious mononucleosis--anorexia,
nausea, asthenia, lassitude, easy fatiguability, and decreased
exercise tolerance demonstrated hepatic involvement in fifteen
sucessive cases of proven infectious mononucleosis. Peterson
(80) and Jordan (56) confirmed his findings of a high per cent
of hepatic involvement present in cases of infectious mono-
nucleosis. Kilham (58) in 1942 demonstrated by liver biopsy
that the histologic lesion was an acute focal hepatitis. Berk,
Shay, Ritter, and Siplet (54) in 1948 performed serial observa-
tions of the behavior of the heterophile antibodies in patients
with infectious hepatitis and on patients with infectious mono-
nucleosis. They were impressed by the hematologic changes sug-
gestive of infectious mononucleosis repeatedly observed in soldiers
with infectious hepatitis (148). The purpose was to discover
whether these phenomena were similar and wherin, they might
differ in these two disorders. Heterophile antibody and absorp-
tion titers were determined after the method of Davidsohn (127).
The authors concluded that "significant increases in heterophile
antibody titer occur only rarely in infectious hepatitis." The
agglutinin in the serum of cases of infectious hepatisis differs
from that in infectious mononucleosis in its absorbability by
guinea-pig kidney. In both infectious hepatitis and infectious
mononucleosis the serum flocculation tests show a marked tendency
to be positive early and to remain positive for long periods It

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is suggested that in infectious mononucleosis, even more than in infectious hepatitis, the flocculation phenomena are associated primarily with alterations in the serum proteins and perhaps lipids. They would appear to be related to liver changes in the disease only insofar as the latter contributes to the alterations in the protein and lipid components of the serum. The authors made note of the fact that the agglutinins in infectious hepatitis were absorbed completely by both guinea-pig kidney and beef erythrocytes. (Therefore they fit the absorption pattern of serum sickness and not the absorption pattern of Forssman heterophile antibodies found in normal sera).

Cohn and Lidman (53) stated that the heterophile agglutinins in their series of patients with infectious hepatitis yielded titers of 1:14 or less but gave no details.

None of the thirty-seven patients with hepatitis developing after mumps convalescent plasma studied by Hawley and his associates (149) showed a heterophile antibody titer greater than 1:14. Eaton, Murphy, and Hanford (144) demonstrated titers of 1:160 of greater in eight per cent of one hundred and fifty cases of post-vaccinal hepatitis and thirteen per cent of sixty eight cases of infectious hepatitis. Leibowitz (126) examined the serum of sixty-five patients with acute virus hepatitis and found an elevation above 1:56 in twenty per cent (thirteen patients) distributed as follows:1:112 in eight cases, 1:224

in four cases, and 1:448 in one case. Havens et al (48) found
three percent of 508 soldiers with viral hepatitis developed
titers of 1:56 which were reduced to 1:7 or negative by absorption
on boiled guinea-pig kidney. Eaton and his corroborators (146)
presented the details of a human liver absorption test which
they believed might prove useful in distinguishing low titer
cases of hepatitis from mononucleosis. Berk et al (54) states
that his experiences with this test in eight cases was
"disappointing".

Schultz (150) selected twelve patients in the terminal
stage of tuberculosis and another twelve who were ready to be
dismissed with the disease completely arrested. In the terminal
group she noted four patients with titers of 1:224, four with
titers of 1:112, and four with titers of 1:28. In the arrested
group she noted two with 1:224, four with 1:112, three with
1:56, and three with 1:28. In all cases there was a positive
titer, and in twenty-seven of forty-five tests on these twenty-
nine patients a "diagnostic" level was found.

C. Neoplasms

The sensitivity of the heterophile antibody test in in-
fectious mononucleosis was known to be faulty early in the
history of the technic. As has been shown, therapy with horse
serum would produce a rise of heterophile antibodies which the
Paul-Bunnell test could not differentiate from that produced in

infectious mononucleosis. Paul and Bunnell themselves (13)
made the comment "...in spite of the fact that the limits of the
reaction which we have described have not been tested, it would
seem to be of diagnostic value. In a sense, however, it seems
to be of more theoretical than practical interest. Theoretical
interest centers about the fact that heterophile antibodies
may be produced or enhanced during the course of human infectious
diseases, and in one disease in particular, in which the etiology
has not been established. Furthermore, that two clinical entities
with widely differing symptomatology such as serum sickness and
infectious mononucleosis would elicit the same type of serologic
response is also worthy of interest... On the other hand it is
also conceivable that the phenomenon which we have described is
in the nature of an isoagglutinin response to the presence of
an excess of abnormal cells either present in the blood or
elsewhere".

Data in the article published by Schultz (150) in 1948
would tend to bear this last supposition out, for all the
diseases which gave diagnostic titers—Hodgkins disease and other
sarcomas, blood dyscrasias and tuberculosis-are diseases charact-
erized by "abnormal cells wither present in the blood or else-
where." She reported sixteen of eighteen tests on six patients
with Hodgkin(s disease in the "diagnostic" range; two of ten
tests on three patients with agranulocytosis in the "diagnostic"
range; while eight tests on two such patients gave no negative

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reactions, but no agglutination over 1:28. Six of eight tests on two patients with monocytic leukemia were reported as "diagnostic". Twenty-four of forty-five tests on six patients with myelogenous leukemia and two of three tests on on patient with polycythemia were reported as being in the "diagnostic" range. To the latter she attached little statistical importance. Nine of twenty three readings on eight patients with sarcoma other than Hodgkins are reported as in the "diagnostic" range.

An explanation for the relatively high antibody titers which Schultz (150) obtained in cases other than infectious mononucleosis lies in the method she used; i.e., readings of the tubes after two hours at room temperature and overnight regrigeration. As a rule this increases the titer by at least one dilution (59,96,104).

Kolmer and Boerner (151) stated as late as in 1945 that "positive reactions do not occur in the leukemias, Hodgkins disease, etc."

Kent (152) reported the case of a fourteen year old boy with leukemia with heterophile antibody titers of 1:4096 and 1:1684 (method of Paul and Bunnell). This patient died and was considered at autopsy to be a dase of myelocytic or monocytic leukemia. Kent raised a question at that time in the title of his report: "False" positive Paul-Bunnell (heterophile) reaction?

Goldman, Fishkin and Peterson (153), employing the technic

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of Stuart, determined the heterophile antibody reactions of 458
miscellaneous cases other than infectious mononucleosis and
found titers of 1:80 or higher in forty-six cases. Fourteen
had titers of 1:160 or higher. They also examined the serum
of twenty-nine persons with Hodgkin's disease and twenty-six
with lympomatous or leukemic states. Three of these fifty-five
patients yielded titers of 1:80 and one had a titer of 1:160.

Carpenter, Kahler, and Reilly (154) reported three instances
of monocytic leukemia in which elevated heterophile antibody
titers were recorded. The technic was not described. These
titers were 1:512, 1:896, and 1:896, respectively.

In the reports of Weinstein and FitzHugh (23) Bernstein
(155), Kent (152), and Schultz (150) the heterophile antibodies
were not characterized.

Raftery and Thompson (156) reported a case of fulminating
leukemia that in the early asymptomatic state was diagnosed as
infectious mononucleosis on the basis of a heterophile titer of
1:448.

Bethell et al (157) cited a report by Etcheverry of a titer
of 1:896 in a case of lymphatic leukemia. The author attributed
the titer to injections of liver which the patient received.
Paul and Bunnell (13) in their original article on the hetero-
phile antibody test found a titer of 1:6400 in a young woman
suffering either from aleukemic leukemia or aplastic anemia,

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whose obscure illness terminated fatally. Bernstein (33) notes
that this patient had received oral liver therapy which he
believed "could hardly account for the elevated titer", although
he states that parenteral liver extracts may produce titers as
high as 1:1280. Southam et al (139) states emphatically that
liver injections are not a cause of elevated heterophile titers
and provide evidence in support of their statement.

Southam et al (139) noted elevated heterophile titers
(1:112 or more) in nineteen per cent of patents with acute
leukemia. They made use of the guinea-pig kidney absorption
technic in their series and noted that the antibody in acute
leukemia is Forssman in nature and could be distinguished from
that which occurs in infectious mononucleosis by use of the
differential absorption test.

They commented that "the possibility of isoimmunization to
autologous antigens seems remote since we were unable to
demonstrate heterophile antigen in normal or neoplastic tissue."

Zarafonetis (158) claimed that the reactions encountered
in luekemias are probably due to cold agglutinins.

D. Rheumatic Fever

Rose, Ragan, Pearce, and Lipman (159) in 1948 published
an article in which they showed that some patients with rheu-
matoid arthritis have a serum factor that causes agglutination
of sensitized sheep cells (sheep erythrocytes mixed with anti-
sheep-erythrocyte serum) at a much greater dilution than it

agglutinates unsensitized sheep cells. This finding was
confirmed by Sulkin et al (160). However, Pike, Sulkin, and
Coggeshall (161) showed that this factor functions not as an
independent antibody, but rather as a potentiating agent for
the heterophile antibody.

XIV. Heterophile Antibody in Spinal Fluid

As early as in 1928, Lohe and Rosenfeld (162) noted that
even when the blood Wassermann is falsely positive, the cerebro-
spinal fluid Wassermann is negative. Slade (163) and Landes,
Reich and Perlow (164) each found a negative spinal heterophile
antibody titer in one case of infectious mononucleosis. Lyons
and Harrison (165) in 1949, after a study of twenty cases of
infectious mononucleosis proved by clinical picture, blood
studies and serologic examination, concluded that the heterophile
antibody of infectious mononucleosis does not pass into the
spinal fluid.

In 1948, however, Silberstein, Bernstein and Stern (178)
demonstrated the presence of heterophile antibodies in cerebro-
spinal fluid from six patients with infectious mononucleosis.
One patient had nervous system involvement, but the titer was
not significantly higher than in the others. They made use of
a large volume of spinal fluid and dilute sheep erythrocte
suspensions as antigen.

Method: "A 1 per cent washed sheep erythrocyte suspension
was prepared and 0.1 ml. added to 1 ml. and 0.5 ml. fresh

spinal fluid in Kahn-sized test tubes and shaken. Saline controls were prepared. The tubes were centrifuged for five minutes at 2,500 rpm, and macroscopic agglutination was demonstrated by shaking the tube. Results were recorded from negative to 4 plus depending on degree of agglutination.

"Davidsohn's absorption method was modified to study the heterophilic nature of the agglutinins found in cerebrospinal fluid. To 1 ml. spinal fluid in one test tube was added 0.5 ml. guinea-pig antigen and to another 0.5 ml. boiled beef erythrocytes. After incubation for 10 minutes at 37 C. the tubes were centrifuged at 2,500 rpm for 10 minutes. One ml. of the supernatant of each was then treated in the manner already described."

Spinal fluid of the six patients with infectious mono-nucleosis was collected the same day as or the day following demonstration of a blood heterophil antibody titer. Hem-agglutinins from 1 plus to 4 plus were present in the spinal fluids of all these patients. They had the same absorption pattern as those in the blood. The serums and spinal fluids of these patients had negative Kahn and Kolmer-Wassermann reactions.

No linear relationship was found between blood and cerebrospinal fluid heterophil antibody titers in these patients during the acute phase of illness. In both the cerebrospinal fluid and the blood sheep cell agglutinins absorbed with boiled

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beef erythrocytes.

XV.   Diagnostic Value of the Heterophile Antibody Test

There are three generally accepted criteria for the diagnosis of infectious mononucleosis; reticuloendothelial hyperplasia, abnormal mononuclear cells, and a positive heterophile antibody agglutination. These, however, are not always encountered together in symptomatic and asymptomatic individuals. The literature is replete with references to the non-specificity of the cells as well as the variability of the heterophile agglutination test. These criteria can be considered as three aspects of the disease; the clinical, hematologic, and serologic. It is the general concensus of clinicians that if any two give positive evidence of the disease, the diagnosis may be considered established.

Kaufman (173) in 1944 observed that no cases in which there was a normal blood count were observed to show positive clinical and serologic evidence of the disease. There were many cases, however, in which there was positive clinical and hematologic evidence of the disease but normal serologic reactions, and a few cases in which there was positive hematologic and serologic evidence, but a subclinical or atypical clinical picture. He concluded that when a sheep-cell agglutination occurs in a dilution of 1:32 with the Paul-Bunnell technic and 1:56 with the Davidsohn technic, it must be considered indicative of the disease, An agglutination in a dilution of 1:16 with the Paul-

Bunnell technic and in a dilution of 1:28 with the Davidsohn technic requires further investigation.

Patients in categories found by Kaufman to give false positive heterophile antibody titers in dilution of 1:56 or greater (Hodgkin's disease, rubella, and liver injections) did not have positive serologic findings after guinea-pig kidney absorption.

Many writers (38,85,96,112,123,139,147,153) affirm the necessity of performing the differential absorption test particularly in cases with borderline titers and in cases complicated by serum injection.

Two editorials (170,171) in 1952 noted that variations in findings of sheep-cell titers in patients with infectious mononucleosis and other diseases are known to take place. It was stated that neither atypical lymphocytes nor sheep-cell agglutination alone should be accepted as positive evidence of infectious mononucleosis, and it was "generally accepted" that, especially in sporadic cases, a characteristic blood picture and preferably a significantly positive blood picture are required for a positive diagnosis.

Zarafonetis (158) stated that "serologic tests are most reliably diagnostic when they reveal a progressive rise in titer in successive serum samples taken during the course of illness and convalescence. The determination of two or more points on the arc of antibody dynamics is usually adequate for diagnostic

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purposes. Occasionally, however two serum specimans may give
identical titers, having caught the antibodies at the same level
first during their rise and then during their fall from an
intermediate peak. In addition a plateau effect may be encounter-
ed, giving rise to similarity of titers in samples taken during
that period." Reference was made to a previous article by him-
self and several co-workers (172).

Regarding the optimum time for obtaining a critical titer,
Kaufman (85) best summarizes the opinion of most investigators:
"If only one diagnostic venipuncture is to be done, it would
seem best to do this not earlier than the nineth day nor later
than the thirtieth day—and preferably between the twelfth and
twenty-first day."

As has been indicated earlier (page 10) there is consider-
able variation in the lowest titer considered diagnostic due to
corresponding variations in technic of performing the heterophile
antibody determination. Since the two tests commonly performed
in this country are the original Paul-Bunnell technic and the
Davidsohn modification, the diagnostic titer of heterophile
antibodies will be considered with reference to those technics.
That even here there may be considerable variation is evidenced
by the statement by Paul (75):

"Ninety per cent of our cases had a positive serology.
This may indicate we are relying too much on the sheep-cell
test for our diagnosis." Kaufman (173) is inclined to agree

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with this interpretation, finding fifty-one at seventy-nine
serologically tested cases difinitely positive (1:56 or more),
eight borderline (1:28 with "correct" differential), and twenty
negative.

Kaufman's assumption that 1:28 with a "correct" differential
constituted a positive test was not shared by many investigators.
Most authors consider the lower level of positivity at 1:32 with
the Paul-Bunnell test and 1:56 with the Davidsohn technic; the
latter figure being considered as positive only after a differ-
ential absorption test reveals little absorption with guinea-
pig kidney and complete absorption with beef erythrocytes. How-
ever, Hoagland (129) considers as a diagnostically critical titer,
one of 1:56 of unabsorbed and 1:28 of guinea-pig kidney absorbed
serum. It appears that the more recent trend, with increasing
use of the differential absorption test, is toward considering such
titers as indicative of infectious mononucleosis. Leibowitz (140)
recommends the use of the absorption tests routinely whenever the
antibody titer is 1:896 or lower, basing his reasons on the
reported indentification of normal Forssman antibodies in diseases
other than infectious mononucleosis at titers up to this level.

Despite almost unanimous acceptance of the differential
absorption test as an unfailing guide in distinguishing low
titers of the heterophile antibodies found in infectious mono-
nucleosis from Forssman antibodies, there have been indications
in the literature (38,96,173,175) that this procedure, also

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has its limitations, and that the absorption test at low titers is not uniformly satisfactory. Kaufamn (173) states that the sera of several patients with infectious mononucleosis gave reactions of serum sickness, and at different courses in the same disease, serum from the same patients gave different qualitative reactions with or without changes in agglutinin titer. Wechsler et al (38) cites two cases presented by Demanche (176) one of which showed no absorption by either antigen, while in the other, affinity for beef red cells took place only after twenty-four hours. Wechsler comments about some cases in his series: "In some cases where guinea-pig kidney failed to completely absorb the agglutinin, beef red cells also failed to do so; and in some of these the latter absorbed a smaller percentage of the agglutinins than did the guinea-pig suspension. In others, although there was no history of serum disease or recent injections of serum, both suspensions completely absorbed the sheep-cell agglutinins. These cases were otherwise indistinguishable from the others in this series both clinically and hematologically, and titers before absorption were occasionally as high as 1:896." No explanation was offered for these phenomena. Perhaps the fact that the normal Forssman antibodies are elevated (page 18) before the development of the typical heterophile antibody of infectious mononucleosis may account for some of the variation. It is also grounds for conjecture that perhaps the heterophile agglutinin of infectious mononucleosis varies in

character with

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the stage of the disease.

The differential absorption test has also failed to reduce the number of "seronegative" cases of infectious mononucleosis. It is noteworthy that the great majority of these "seronegative" cases are reported as occurring in epidemics (37,38,82,94,177, 179).

Inasmuch as person to person transmission of mononucleosis is almost unknown (180) and experimental transmissions have failed to reveal cases meeting adequate diagnostic criteria (181), it is quite possible that these epidemics may possibly be instances of a disease, or diseases, related to but not identical with infectious mononucleosis.

Despite the fact that Davidsohn and Walker (25) and Stuart, Fulton, Ash and Gregory (109) had shown that the antibodies in infectious mononucleosis differed from the Forssman type, the role of ox cells in the serologic diagnosis was relegated to that of confirmation. Bailey and Raffel (106) and Stuart, Griffen, Wheeler and Battey (182) demonstrated that the hemolytic titer to ox cells was greatly raised while the agglutinin titer was scarcely affected. Gleeson-White, Heard, Mynors and Coombs (183) confirmed this finding, and showed that, whereas the cells of individual oxen reacted variably to agglutination, all could be hemolysed to the same degree. Beer (111) and Foord and Butt (30) performed ox cell hemolysin tests on a few cases,

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generally confirming expected results.

No further references to the use of direct ox cell antibody estimations in infectious mononucleosis could be found until in 1951, when Mason (174) proposed an ox cell hemolysin test for the diagnosis of infectious mononucleosis.

Since naturally occurring ox cell antibodies are normally absent, or present in very low concentration, and are not known to be raised in any conditions other than infectious mononucleosis and serum sickness, a single tube test was devised which permitted the testing of large numbers of sera and which compared favorably with the results obtained by the sheep-cell agglutination and differential absorption test of Davidsohn.

The results in sixty cases of infectious mononucleosis and 200 controls were evaluated, and it was found that the ox cell hemolysin test did not significantly reduce the proportion of serologically negative cases, but often confirmed the diagnosis before the characteristic sheep cell agglutins were demonstrable.

The test was found to be as sensitive and as specific as other serologic tests for infectious mononucleosis and was much more easily performed.

For details of the technic of this single-tube ox-cell hemolysin test for infectious mononucleosis, the reader is referred to the original article (174).

XVI. Summary

A. A brief introduction is given covering the pertinent points

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in the history of infectious mononucleosis and of heterophile
antibodies prior to the discovery in 1932, by Paul and Bunnell,
of increased heterophile antibodies in infectious mononucleosis.

At the time of the earlier investigations considered in
this thesis, infectious mononucleosis was not considered by
many to be a definite disease entity. Means of diagnosis were
unsatisfactory and clinical and hematologic manifestations varied.
B. The technic of the heterophile antibody test proposed by
Paul and Bunnell in 1932 is briefly considered.
C. Certain modifications of this test result in a more sensitive
procedure. Quick methods make use of microscope slide-tests
and centrifugation. A rapid screening test for infectious
mononucleosis is described.
D. Due to the non-specificity of the heterophile antibody test,
there are wide variations in the lowest titer considered diag-
nostic; values range from 1:8 to 1:512. In 21 reported series
totalling 1,643 cases taken from the literature the percentage
of positive serologic tests averaged approximately 83 per cent.
E. Investigators predominantly agree that a negative heterophile
antibody test, even though the test is repeatedly negative,
does not rule out the diagnosis of infectious mononucleosis.
Epidemics of infectious mononucleosis, though rarely reported,
tend toward unsatisfactory serologic findings. Reports of
"seronegative" cases of infectious mononucleosis are considered.
F. Common sources of error leading to the wide variations in

reports found in the literature are presented. These variations
stem primarily from the fact that a standard laboratory method
for determining sheep cell agglutination has not been used in
the evaluation of the heterophile antibody titer in infectious
mononucleosis. There are certain errors inherent in the test
itself, but these can be avoided.

Grounds on which "seronegative" cases are reported are
criticized.

G. Definition and occurrence of heterophile antibodies is
presented. Evidence leading to the postulation of three types
of sheep cell agglutinins is given.

H. Reports of occasionally occuring false-positive Wassermann
reaction in patients with infectious mononucleosis are reviewed.
The apparent relationship between sheep-cell antibodies and
these false-positive serologies does not exist in fact.

I. Review is made of the publication of Davidsohn in 1937
wherein he presents a differential absorption test as a means
of increasing the sensitivity of the heterophile antibody de-
termination. Principles underlying this test are presented and
evidence of its particular value in certain instances is given.

J. A proposed modification of the Davidsohn differential-
agglutination test is presented.

K. The range of normally-occurring Forssman type heterophile
antibodies is given. A review is made of the various reports

in which these antibodies were reported. A titer of 1:56 maybe considered the upper limits of normal in an apparently healthy adult when Davidsohn's technic is used.

L. With few exceptions, the heterophile antibody test becomes positive, if it is to become so at all, by the end of the second week of illness. The test usually remains positive for two to four months, and rarely persists beyond six months after its initial appearance. The usual titer ranges between 1:320 and 1:10,240.

M. Increased heterophile antibody titers, giving a "diagnostic" sheep-cell agglutinin titer are encountered relatively commonly in serum therapy, in infectious hepatitis, in tuberculosis, and in various neoplasms, particularly the lymphomas, lymphatic and myelogenous leukemia. The sheep cell agglutination in rheumatic fever is considered to be due to an augmentation of the normally occurring heterophile agglutinins.

N. There appears to be some controversy as to the presence of heterophile antibodies in the spinal fluid of patients with infectious mononucleosis. Only one investigation was found which revealed their presence. The method used in this instance is presented. There was no linear relationship found between the blood and spinal fluid heterophile antibody titers during the acute phase of the illness. Absorption tests revealed them to be the typical infectious mononucleosis antibodies.

O. There are three generally accepted criteria for the diagnosis
of infectious mononucleosis; reticuloendothelial hyperplasia,
abnormal mononuclear cells, and a positive heterophile aglut-
ination. These criteria are individually represented as three
aspects of the disease; the clinical, the hematologic, and the
serologic. If any two give positive evidence of the disease,
the diagnosis may be considered established.

Due to the occasional increase in normally-occurring
Forssman-type heterophile antibodies in conditions other than
infectious mononucleosis, it is generally agreed that a diag-
nosis of infectious mononucleosis should be made only after
absorption tests are carried out. The current trend is toward
considering as a diagnostically critical titer one of 1:56
of unabsorbed and 1:28 of guinea-pig kidney absorbed serum.

There have been occasional reports of the differential
absorption test, at low titers, giving equivocal results. It
is suggested that this fact, among others, may indicate that
the heterophile agglutinin of infectious mononucleosis varies
in character with the stage of the disease or differs somewhat
in epidemics from that in sporadic cases.

·   Since the heterophile antibody in infectious mononucleosis
differs from the Forssman-type antibody found in other conditions
chiefly in its relationship to absorption with ox erythrocytes,
it is puzzling that experimentation has not been more extensively

performed using ox cells rather than sheep cells as the basis
of a serologic test. Mention is made of a proposed ox-cell
hemolysin test for the diagnosis of infectious mononucleosis,
in which a single tube titer may be used for a positive diagnosis.
XVII. Conclusion:

Heterophile antibodies are elevated in patients with in-
fectious mononucleosis. This elevation is more consistently
present in isolated cases than in reports of epidemics. This
elevation of heterophile antibodies may be demonstrated by
serologic tests embodying the agglutination of sheep erythro-
cytes by the heterophile antibodies, which function as agglutinins.
The two most common technics used in this country are those of
Paul and Bunnell, and of Davidsohn. The sheep cell agglutinins
in infectious mononucleosis differ from those found after serum
injection and in certain other conditions. They may be differ-
entiated by means of a differential absorption test advocated
originally by Davidsohn.

The heterophile antibodies of infectious mononucleosis as
differentiated by the absorption test, are specific for this
disease. They usually occur in diagnostically critical titers,
when such titers occur, by the end of the second week of illness;
and usually remain elevated for two to four months. The usual
titer ranges between 1:320 and 1:10,240. A diagnostic titer is
considered as one in which the agglutinin titer before absorption

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is 1:56 or more, and after absorption with guinea-pig kidney, is 1:28 or more.

The differential absorption test is known to occasionally give equivocal readings in cases with borderline titers. Also, there have been exceptionally high percentages of "seronegative" cases of infectious mononucleosis reported in epidemics of the disease. Conjecture is made as to whether the heterophile agglutinin of infectious mononucleosis varies in character with the stage of the disease or differs somewhat in epidemics from that in sporadic cases.

To the previously accepted diagnostic criteria of reticulo-endothelial hyperplasia and abnormal mononuclear cells, must now be added serologic confirmation of the disease by a positive differential absorption test. It is generally accepted by investigators that if any two of the three aspects of this disease (clinical, hematologic, serologic) are present, the diagnosis of infectious mononucleosis may be made. This consideration was justified while the serologic phase of the disease was in an experimental stage. With almost unanimous acceptance of the differential absorption test as being specific and sensitive for the disease, it now joins the ranks of the other two criteria as equal or superior in diagnostic import.

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