Inhibition of Vaccinial Hemagglutinins by Sera of Patients with Coronary Heart Disease and Other Chronic Illnesses

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Because of evidence relating disturbances in lipid metabolism and transport to atherosclerosis, it seemed of interest to investigate the behavior of sera from affected subjects with a serologically active lipid. This interaction might be reflected in alterations in the serum and thus permit detection and quantitation of the disturbance. Vaccinial hemagglutinin (VHA) appeared to be a likely material for this investigation since it is a lipoprotein complex, readily quantitated by hemagglutination, and known to react in vitro with some serum substances.\(^1\)\(^2\) Inhibition of hemagglutination by these substances is usually presumed to represent specific antibody, formed in response to vaccination or natural infection;\(^3\)\(^4\) however, nonspecific inhibition has also been described in animal sera and in human pathological body fluids.\(^5\)\(^6\) It is suggested that alterations in the level of nonspecific inhibition might be indicative of the metabolic disturbances related to atherosclerosis.

**Methods**

**HEMAGGLUTININ**

Vaccinial hemagglutinin was prepared by infecting four-day Hela cell cultures with a standard pool of virus diluted to contain \(5 \times 10^7\) egg plaque forming unit (PFU) per ml. After removing the overlying growth medium and rinsing the cell sheet with balanced salt solution, maintenance medium (Sherer’s maintenance solution (MS) + 10 per cent horse serum) containing the diluted virus was allowed to remain in contact with the cell sheet for four hours. After this interval, the virus suspension was removed, the cell sheet rinsed twice with balanced salt solution and fresh maintenance medium added. Thirty hours after inoculation, the cells still adherent to the glass were scraped into the overlying fluid medium. This suspension was centrifuged at low speed to separate the cells which still contained most of the hemagglutinin.

The cells were disrupted mechanically in a tissue homogenizer or by sonic vibration in a volume of sterile citrate-saline (0.05 M citrate in 0.1 M NaCl) equal to one-tenth that of the original suspension. This concentrated cell homogenate was centrifuged at low speed to remove large aggregates of cellular debris, and the resulting opalescent fluid served as the source of vaccinial hemagglutinin. The hemagglutinating titer was usually 1:160, although individual lots varied in titer from 1:40 to 1:640.

Normal Hela cell cultures and cultures infected with another virus (herpesvirus) were treated in the same manner and failed to show any hemagglutinating activity with chicken red cells sensitive to lipid hemagglutination.

**CHICKEN RED CELLS**

For routine use, red blood cells were obtained from a laboratory stock of white leghorn chickens, whose red cell sensitivity to VHA had been predetermined. A portion of the stock suspension of cells was removed for the day’s use, washed three times in 0.15 M NaCl, packed, and made up as a 10 per cent suspension. This suspension served as stock for the final cell suspension of 0.5 per cent concentration.

**SERA**

After removal from the clot with aseptic precautions, human test sera were kept at -20 C., in several aliquots when possible. No preservative was added. For use in the hemagglutination-inhibition test (HI), the sera were routinely heated at 56 C. for 30 minutes in a 1:10 dilution in 0.15 M NaCl to inactivate complement. Occasional sera had a significant degree of nonspecific agglutinating activity for the red cells, which could be removed by preadsorption of the initial 1:10 dilu-
VAOOINIAL HEMAGGLUTININS AND HEAET DISEASE

FIGURE 1
Distribution of vaccinia hemagglutinin inhibitory titers in sera of patients by age and sex.

Standardization of the Hemagglutinin

Two units of VHA contained in 0.25 ml. of saline were used. The dilution to be used was determined by a titration of the hemagglutinin; the highest dilution of antigen, 0.25 ml. of which
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TABLE 2

Distribution of Vaccinia Hemagglutinin Inhibitory Titters in Patients with Chronic Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Per cent</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>102</td>
<td>100.0</td>
</tr>
<tr>
<td>Hypertensive, cerebrovascular, and renal diseases</td>
<td>29</td>
<td>100.0</td>
</tr>
<tr>
<td>Diabetes and other endocrine disorders</td>
<td>20</td>
<td>100.0</td>
</tr>
<tr>
<td>Neoplasms</td>
<td>20</td>
<td>100.0</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>8</td>
<td>100.0</td>
</tr>
<tr>
<td>Chronic infectious diseases</td>
<td>2</td>
<td>100.0</td>
</tr>
<tr>
<td>Rheumatic disease</td>
<td>4</td>
<td>100.0</td>
</tr>
<tr>
<td>Miscellaneous disorders</td>
<td>14</td>
<td>100.0</td>
</tr>
<tr>
<td>Subtotal (excluding coronary heart disease)</td>
<td>97</td>
<td>100.0</td>
</tr>
<tr>
<td>Total all diseases</td>
<td>199</td>
<td>100.0</td>
</tr>
</tbody>
</table>

would result in formation of a complete shield of agglutinated cells, was defined as one hemagglutinin unit.

**Serum Dilutions**

The inactivated sera were diluted in serial twofold dilutions from 1:10 through 1:640 and dispensed in 0.25 ml. amounts to be tested with vaccinia hemagglutinin.

Duplicate tubes for the three lowest dilutions (1:10, 1:20, and 1:40) receiving saline in place of hemagglutinin served as serum controls for nonspecific reactions with the cells.

**Test Procedure**

Two units of VHA in 0.25 ml. were added to each set of serum dilutions. The racks were shaken thoroughly and the VHA-serum mixture incubated at 35 to 37 C. for two hours. After the cell suspension was added, the racks were re-shaken and allowed to stand at room temperature until the cells settled out in a readable pattern. The inhibitory titer of the serum was the highest dilution of serum completely inhibiting agglutination. The usual controls for each hemagglutination were carried out (i.e., check titrations of the dilution of hemagglutinin used were always included, as well as positive sera with a known titer, a serum negative for inhibition of vaccinia hemagglutinin, and several sets of tubes with saline and cells alone as a check on the cells).

**PATIENTS AND CONTROLS**

Sera from patients with various chronic diseases were collected from four different hospitals (table 1). The conditions as determined from the clinical records include coronary and other cardiovascular diseases, diabetes, neoplasms, and a number of other disorders (table 2). A considerable proportion of individuals had multiple diagnoses; in these instances, the major diagnosis is listed. Since there was particular interest in coronary heart disease, a special effort was made to collect sera from such patients; of the 115 persons in this category with a clear history of myocardial infarction, 56 were outpatients and 29 were hospitalized for a condition other than acute myocardial infarction, so that patients recovering from the acute episode were excluded. All other patients, apart from 4 persons with diabetes, were hospitalized at the time the blood specimen was obtained.

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FIGURE 2
Distribution of vaccinia hemagglutinin inhibitory titers in sera of patients with and without coronary heart disease (CHD) by age and sex.

To determine whether the titers of sera from these patients differed in distribution from those in the general population, a control series was run on a 10 per cent random sample of households in a small city (Tecumseh, Michigan). The sera were collected in the course of an extensive epidemiological study of health and disease in this natural community. Sera were available on approximately 90 per cent of all persons four years and older in the sample, a total of 691 specimens (table 1). This population sample included 10 men and 3 women with a diagnosis of "probable" coronary heart disease, based on the presence of a history of myocardial infarction, angina pectoris, characteristic electrocardiographic abnormalities, singly or in combination.

STATISTICAL METHODS
Statistical analysis of the data was performed using the chi-square test.
Results

Vaccinia hemagglutinin inhibitory (VHAI) titers in the upper range (1:20 and over) occurred in 164 of the 279 patients with chronic diseases, an overall frequency of 61 per cent in men and 53 per cent in women (table 2). Detailed analysis of these distributions by age and sex (fig. 1) indicated neither any age trend in the two sexes \((P > 0.3\) for men; \(P > 0.2\) for women), nor any sex differences at any age \((P > 0.3\) to \(< 0.5\) in the various age ranges). There was, however, a tendency toward lower titers at age 75 and over. In the age range 30 to 74, the group most suitable for comparison with the control population to be described later, the frequency of elevated titers was 63 per cent in men and 56 per cent in women (table 3).

Comparison of the distributions among men with coronary heart disease and men with chronic disorders other than coronary heart disease (fig. 2) revealed no significant difference in the combined age group 30 to 74 \((P\) between 0.2 and 0.1). The number of women with coronary heart disease was insufficient for a similar analysis. Nevertheless, when the comparison was made between all persons with and without coronary heart disease, regardless of age and sex (fig. 3), those with this diagnosis showed significantly higher titers \((P\) between 0.02 and 0.01). The distributions among men and women without coronary heart disease did not differ \((P > 0.9)\) (fig. 2).

The data presented thus far indicate a high prevalence of elevated VHAI titers among men and women with chronic disorders, with a suggestion that the tendency is particularly marked among patients with coronary heart disease. These impressions gain support on comparing these distributions with those found in a sample of a general population.

In contrast to the patients described, VHAI titers in the upper range occur with less frequency in the population at large (fig. 4). Thus, no more than 40 per cent of the men and 36 per cent of the women aged 30 to 74 in the general population sample had titers above 1:10 as compared with 63 and 56 per cent, respectively, among the men and women with chronic diseases (table 3). Higher titers
were somewhat more common at ages 4 to 29, but the difference was not statistically significant; the slightly higher levels at younger ages presumably reflect residual specific immunity from vaccination in childhood or military service. Beyond age 60, about two-thirds of the general population showed titers of 1:10 or less (table 3). Statistical analysis demonstrated no significant sex difference in titer distribution at any age level or in the total group. If it is true that persons with some of the chronic disorders tend to have elevated VHAII titers, it follows that the distributions shown for the general population, including such individuals, must be skewed toward the high side. Unfortunately, the sample did not include an adequate number of persons with more advanced degrees of disorder to permit valid comparisons between

### TABLE 4

**Distribution of Vaccinia Hemagglutinin Inhibitory Titers Compiled from Various Sources**

<table>
<thead>
<tr>
<th>Group</th>
<th>Status</th>
<th>Number</th>
<th>Titer &lt; 1:20</th>
<th>Titer 1:20 or &gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number</td>
<td>Per cent</td>
</tr>
<tr>
<td>1. Various</td>
<td>Vaccinated once—response to vaccination:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(titers before re vaccination)</td>
<td>Primary</td>
<td>12</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Accelerated</td>
<td>31</td>
<td>27</td>
<td>87.1</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>15</td>
<td>12</td>
<td>80</td>
</tr>
<tr>
<td>2. Mothers</td>
<td>Vaccinated less than 3 years ago</td>
<td>102</td>
<td>7</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>Vaccinated more than 3 years ago</td>
<td>89</td>
<td>49</td>
<td>56.1</td>
</tr>
<tr>
<td>3. Various</td>
<td>Revaccinated more than 3 years previously</td>
<td>79</td>
<td>67</td>
<td>84.8</td>
</tr>
<tr>
<td>4. Various</td>
<td>Revaccinated more than 2½ years previously</td>
<td>28</td>
<td>23</td>
<td>89.3</td>
</tr>
<tr>
<td>5. Adults: 23-27 years</td>
<td>Vaccinated 2½ to 16 years previously</td>
<td>19</td>
<td>15</td>
<td>78.9</td>
</tr>
<tr>
<td></td>
<td>(1:8 or &lt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1:16 or &gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 1-5</td>
<td></td>
<td>366</td>
<td>214</td>
<td>58.5</td>
</tr>
<tr>
<td>6. A 10 per cent random sample from a population</td>
<td></td>
<td>691</td>
<td>404</td>
<td>58.5</td>
</tr>
<tr>
<td>7. Adults</td>
<td>Vaccinated once or more than once</td>
<td>1040</td>
<td>269</td>
<td>25.2</td>
</tr>
</tbody>
</table>

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"healthy" and "diseased" persons within the sample.

To summarize the data, all age groups between 30 and 74 were combined; this was justifiable in view of the demonstrated absence of age trends. The difference in titer distribution (fig. 5) between the group of men with coronary heart disease and men in the population sample was highly significant \( (P < 0.001) \); the difference between men with diseases other than coronary heart disease and men in the population sample was close to significance \( (P \text{ between } 0.1 \text{ and } 0.05) \). On the other hand, men with and without coronary heart disease in the "chronic disease" group did not differ significantly in titer distribution \( (P \text{ between } 0.2 \text{ and } 0.1) \). Sera of women with chronic disease differed significantly in titer distribution from those in the general population \( (P < 0.01) \) (fig. 6).

In view of the statistical association between serum cholesterol levels and coronary heart disease, VHAI titers were compared with serum cholesterol levels in the general population sample. Average serum cholesterol levels appeared to be similar in persons of the same sex and comparable age, regardless of titer. Conversely, geometric mean titers
showed no apparent variation at different ranges of serum cholesterol, grouped by age and sex. A lack of a direct relationship between inhibitor level and serum lipid content is also suggested by our observation that complete removal of lipoproteins from serum by ultracentrifugation at density of 1.21 will not alter the titer of the infranatant.

Discussion

To assess the significance of the present data, it is important to ascertain whether persistently elevated VHA1 titers are the result of past vaccination or reflect in part the presence of a serum component associated with a variety of chronic diseases. Preliminary experiments using column chromatography, electrophoresis, and various enzymatic or physical methods do, in fact, indicate that part of the serum inhibitor resides in the gamma globulin fraction, presumably representing specific antibody to vaccinia hemagglutinin. Another inhibitor can, however, be demonstrated in the beta or alpha-2 globulin fraction. Because of the significantly different distribution of VHA1 titers in patients with chronic disease and of the isolation of an inhibitor in the nongamma fraction of serum, it seems unlikely that elevated titers in these patients merely reflect unusually high levels of specific immunity to vaccinia hemagglutinin.

Even though earlier reports of VHA1 titers have assumed, with one exception, that the inhibitor is a specific antibody, it is of interest to compare these data with the present findings, making allowance for variations in serological techniques. It will be noted that the combined data from five studies (table 4) indicate titers of 1:10 or less among 58 per cent of 366 persons, in close agreement with the distribution in our general population sample. In a separate study of 1,040 persons (table 4), the stated titer ranges and groupings are not comparable.

The current investigation is based on the premise that different biological stresses call forth tissue responses which result in the appearance of reactive components detectable by appropriate serological tests. Indeed a limited but careful study showed that cellular injury induced by vaccination can cause the production of such a pair of reactive components, one, a nongamma globulin serum component, the other, conventional antibody in the gammaglobulin fraction (unpublished observations). Conceivably, cellular injury induced by processes other than vaccination causes release of similar nongamma globulin reactants. Furthermore, cellular injury associated with a derangement of lipid metabolism, as in atherosclerosis, might cause the appearance of a lipoprotein which reacts with the serum component described in this investigation. This is all the more plausible since vaccinia hemagglutinin, also a lipoprotein, is not a component of the virus but a lipid product of the infected cell. According to the basic premise, it would share some common properties with an analogous lipoprotein present in patients with cardiovascular and some other chronic diseases. The failure to relate inhibitory titers with serum cholesterol levels or the lipoprotein fraction of serum in no way contradicts the thesis associating the
Vaccinia hemagglutinin inhibitory titers in sera of individuals with and without chronic disease.

Serum inhibitor with a lipid disturbance since the inhibitor is thought to interact with a lipoprotein rather than being a lipoid substance itself. The inhibitor, the distribution of which is discussed, is not, in the classic sense, an antibody but appears to be a serum component developed as a consequence of tissue injury caused by chronic disease and capable of reacting with its lipid products or the analogous lipoprotein vaccinial hemagglutinin.

Further work is being done on the biochemical identification of the inhibitor and relation between total inhibitory titer, as reported here, and the titers residing in the various serum fractions. The estimation of titers in fractions rather than total serum may possibly increase the sensitivity of the test, i.e., reduce the "false negatives." Consideration of the specificity of the test draws attention to the considerable number of "false positive" results found in the general and overtly normal population. It would not seem likely that refinement of the test procedure will materially reduce the number of positive titers of unexplained origin. Rather, the frequency of elevated titers in the population at large suggests that a positive test in a manifestly healthy individual may indicate the presence of preclinical disease or, at least, a tissue response characteristic of disease-provoking stresses, apart from vaccination. The serological recognition of coronary and other arteriosclerotic disorders along the line suggested by this study has not only practical usefulness but possibly affords new insights into etiological factors.

**Summary**

Serum inhibitory titers to the agglutination of chicken red cells by vaccinial hemagglutinin have been measured in persons with a variety of chronic diseases and in a control sample of the general population. Between the ages of 30 and 74, elevated titers occurred in 63 per cent of the men and 56 per cent of the women with chronic diseases, but in only 40 per cent of the men and 36 per cent of the women in the "control" population. Patients with coronary heart disease tended to show the highest titers. The suggestion is made that the serum inhibitor under study may reflect, in part, relatively "nonspecific" pathological changes and tissue responses provoked by various biological stresses and is a promising approach to the recognition of cardiovascular disease and related disorders.

**Acknowledgment**

Sera from patients with chronic diseases were obtained through the cooperation of Dr. Bernard A. Beren, Wayne County General Hospital; Dr. Ralph L. Brandt, Washtenaw County Home; Dr. William D. Robinson, Department of Internal Medicine, University of Michigan Medical School; and Dr. Henry K. Schoch, Veterans Administration Hospital, Ann Arbor, Michigan. Dr. Richard D. Remington gave advice on the statistical analysis.

**References**

VACCINIAL HEMAGGLUTININS AND HEART DISEASE


Erratum

Vol. X, page 91: Equations given in the footnote are incorrect and could not be used to adjust the clearance rates for the variations in viscosity with temperature. The data in table 1 were obtained by the simple correction:

\[ K_c = K_t \times \frac{V}{V_{37}} \]

The symbols here are as identified in the original footnote.
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