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IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF THE INFECTIOUS HEPATITIS (HEPATITIS A) AGENT

Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

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IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF THE INFECTIOUS HEPATITIS (HEPATITIS A) AGENT

by

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. In Cincinnati, the Environmental Research Center possesses this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

The Health Effects Research Laboratory conducts studies to identify environmental contaminants singly or in combination, discern their relationships, and to detect, define, and quantify their health and economic effects utilizing appropriate clinical, epidemiological, toxicological, and socio-economic assessment methodologies.

Enteric viruses are an environmental contaminant that are found in the feces of infected individuals and in sewage. The virus(es) of infectious hepatitis (Hepatitis A) is a member of the enteric virus group and is the one member that has been conclusively shown to be transmitted via water. The virus(es) have proven very difficult to characterize in the laboratory and this study was designed to combine the techniques of electron microscopy, ultra centrifugation, column chromatography, tissue culture and serology to isolate, identify and characterize the causative agent(s).

R. J. Garner
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ABSTRACT

This research program has had the overall objective of combining the techniques of electron microscopy, ultracentrifugation, column chromatography, tissue culture and serology to identify, isolate and characterize the infectious hepatitis (hepatitis A) etiologic agent, to propagate it in cell cultures and to develop in vitro immunodiagnostic assays capable of detecting the presence of the hepatitis A virus and its antibody both in the patient and the environment.

Using program results, it was possible to relate this agent morphologically and serologically to those isolated from other, geographically separated hepatitis A epidemics. Employing hepatitis A antigen detected by radioimmunoassay screening and isolated from stool specimens collected from patients with either clinical or subclinical hepatitis, a radioimmunoassay and an immune adherence hemagglutination assay were developed for the detection of antibody to hepatitis A (anti-HA). Using these assays, the protective effect of circulating anti-HA against reinfection and the prophylactic effect of commercial immune serum globulin containing anti-HA were established. Likewise, a survey of a commercial plasma donor population was conducted to obtain information on the distribution of anti-HA among the population as a whole. It was found that approximately 37% of the tested donors had anti-HA, the frequency varying directly with age.

All attempts to propagate the hepatitis A virus in tissue culture have been unsuccessful.

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ABBREVIATIONS AND SYMBOLS

anti-HA	-- antibody to hepatitis A
cpm	-- counts per minute
EDTA	-- ethylenediaminetetraacetic acid
g/cm^3	-- grams per cubic centimeter
GVB	-- gelatin veronal buffer
HAAg (HAV)	-- hepatitis A antigen (hepatitis A virus)
hr	-- hour
IAHA	-- immune adherence hemagglutination assay
^{125}I anti-HA	-- antibody to hepatitis A radioactively labelled with 125-iodine
IEM	-- immune electron microscopy
IgG	-- gamma globulin
ISG	-- immune serum globulin
KPO_4	-- potassium phosphate ion
µCi	-- microCurie
µl; µg	-- microliter (0.001 ml); microgram (0.01 g)
ml; mg	-- milliliter; milligram
min	-- minute
MS-1	-- viral hepatitis type A
MS-2	-- viral hepatitis type B
$(NH_4)_2SO_4$	-- ammonium sulfate
PBS-FCS	-- phosphate buffered saline - fetal calf serum
RIA	-- radioimmunoassay
SGPT	-- serum glutamic pyruvic transaminase
w/v	-- weight to volume
P/N	-- positive cpm ÷ negative control cpm

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SECTION I

INTRODUCTION

Two forms of hepatitis have been described on the basis of clinical, epidemiological and immunological features (1). Infectious hepatitis, or viral hepatitis type A (MS-1), has an average incubation period of about 30 days and is generally spread by direct contact, e.g. fecal-oral transmission, or by fecal contamination of drinking water or food. Serum hepatitis, or hepatitis type B (MS-2), on the other hand, has an average incubation period of about 90 days and is usually, though not necessarily, transmitted through parenteral routes (2-4). Following the initial report of Blumberg et al. (5) of the existence of the Australia antigen, a definite link was established by several investigators between serum hepatitis and this antigen. Subsequently much detailed information has been published on morphological (6-10), biophysical (11-13), biochemical (14) and immunological (15, 16) characteristics of the hepatitis B antigen (HBAg).

This has not been true, however, of the hepatitis A antigen (HAAg). Since the report of Feinstone et al. (17), several investigators have confirmed the role of a 27 nm particle in the etiology of hepatitis A (18-20) and some morphological and biophysical studies have appeared (21-26), but the replicative cycle, distribution, biochemistry and immunological complexity of the virus are still relatively unknown. Because of the general unavailability of HAAg in quantities necessary for detailed research, much of this information must remain unknown until reliable, affordable and reproducible in vitro and/or in vivo hosts (chimpanzees and certain marmoset species) favor the development of in vitro techniques, all of which, thus far, have been found unsuccessful.

Although few deaths occur as a direct result of infectious hepatitis, over 250,000 cases have been reported in the United States during the past 5 years (27). This figure is thought to represent about 10% of the actual cases (28), which result in enormous financial costs due to lost work time, medical expenses and general debilitation. In 1973, the World Health Organization speculated that approximately 10% of the people swimming in the Mediterranean Sea that year contracted hepatitis.

Because, according to published scientific literature, HAAg has generally been found only in stools of icteric hepatitis patients, and then only during a very restricted period of less than two weeks prior to peak aminotransferase elevation (20, 23), the virus has usually disappeared before actual diagnosis of the disease. Thus an adequately thorough and controlled sample population is rare and valuable for

detailed studies.

Such a population has been available to us from the epidemic of hepatitis A that occurred in 1970 at the Lynchburg (Va.) Training School and Hospital (29). This epidemic produced within five months approximately 565 cases of hepatitis A in a population of 3600 mentally retarded. From these, special attention was given to a group of 473 severely retarded patients, 57% of whom contracted clinical hepatitis A. Between June 1970 and February 1971, several thousand serum samples were collected, mostly from the special study group. Blood was drawn from these 473 patients at least bi-weekly during the period of their illness and several times during a 6-month follow up. Infrequent or single serum specimens were collected from 727 other patients in the institution during the epidemic. Thus, serial specimens were accumulated of pre-infection, acute and convalescent sera, along with negative controls. In addition, approximately 450 stool samples were obtained. Serum glutamic pyruvic transaminase (SGPT) determinations were made on all the sera at the time when the samples were originally collected.

Since January 1, 1975, we have been examining the Lynchburg serum and fecal samples:

1. to identify, isolate and characterize the infectious agent (HAAg) involved in this hepatitis A epidemic;

2. to relate it morphologically and serologically to the causative agent of similar geographically isolated hepatitis A epidemics;

3. to determine its pattern of occurrence within a patient during the course of disease;

4. to determine its distribution throughout the general population, as indicated by the frequency of antibody to hepatitis A (anti-HA);

5. to determine the course of development of anti-HA and its protective effect against further infection by HAAg;

6. to determine the prophylactic effect of commercial immune serum globulin in treating a population exposed to HAAg;

7. to develop <u>in vitro</u> diagnostic immunoassays for the detection of hepatitis A antigen and antibody.

This report contains a description of the specific investigations carried out between January 1, 1975 and June 30, 1977 in an effort to attain these objectives.

SECTION 2

CONCLUSIONS

Investigations supported by Grant No. R-804003 have enabled us to accomplish the following:

1. show the morphological and serological similarity of the Lynchburg hepatitis agent to MS-1 (1), the Joliet agent (30), the Phoenix agent (22), the Costa Rican (and marmoset) agent (18, 24) and German agent (31);

2. develop a microtiter radioimmunoassay for the detection of hepatitis A antigen (32);

3. establish the reliability of RIA screening of fecal samples for HAAg by comparing this technique with immune electron microscopy (32);

4. adapt an immune adherence hemagglutination assay for the detection of antibody to hepatitis A utilizing fecal HAAg prepared without the need for involved centrifugal and chromatographic purification (33);

5. survey sera from the most heavily exposed and infected Lynchburg wards for distribution of anti-HA and thus demonstrate the protective effect of naturally occurring antibody against reinfection (34);

6. show the protective effect of immune serum globulin, provided it was administered prior to exposure to the virus (35);

7. show that anicteric as well as icteric patients can excrete antigen in their stools (35);

8. show that HAAg excretion can begin soon after exposure and continue beyond peak transaminase elevation (36);

9. demonstrate the existence in experimental animals (marmoset) of an asymptomatic "antigen carrier" and suggest the possibility of such a carrier among human populations (37);

10. demonstrate the feasibility of a surveillance program in institutions for the mentally retarded, whereby the presence or absence of anti-HA could be known by regular monitoring of each patient's

serum. Such a program could provide a better means of preventing hepatitis epidemics in such institutions and would greatly aid in proper prophylaxis in emergency situations (35);

11. suggest minimum standards for commercial immune serum globulin destined for use in the treatment or prevention of hepatitis A. This is now possible with the existence of assays for anti-HA (38).

SECTION 3

MATERIALS AND METHODS

POPULATIONS TESTED

Human

From April to September 1970, 375 clinical and 190 sub-clinical cases of hepatitis A were recorded among 3600 patients at the Lynchburg (Virginia) Training School and Hospital, an institution for the mentally retarded (29). Between June 1970 and February 1971, several thousand serum samples were collected, mostly from a special study group of 473 severely retarded patients, of whom 57% contracted clinically apparent hepatitis A. Blood was drawn from these 473 patients bi-weekly during the period of their illness and several times during a six month follow up. Infrequent or single serum samples were collected from 727 other patients in the institution during the epidemic. Thus, there were accumulated serial specimens of pre-infection, acute and convalescent sera, along with negative controls. In addition, several stool specimens were also obtained from patients in the ten wards most seriously affected by the epidemic. Since stools were collected randomly over the course of the epidemic both from patients with and without the disease, it turned out that the collection contained specimens from all stages of infection, as well as several negative controls.

Marmoset

Six adult white-lipped marmosets (Saguinus fuscicollis, S. nigricollis) were inoculated intramuscularly with 0.5 ml of a 6.6% (w/v) human fecal suspension containing hepatitis A virus, as demonstrated by immune electron microscopy. The original fecal sample was collected by G.G. Frösner (31) in Gomaringen, Germany, and sent to several laboratories for IEM identification. Finally it was sent to Drs. F. Deinhardt and D. Peterson (Rush-Presbyterian-St. Luke's Hospital, Chicago, Illinois) for infectivity studies in marmosets. Prior to the commencement of the experiment, several fecal and serum samples were collected from each animal to serve as negative controls. From the day of inoculation, stools were collected daily, serum weekly for transaminase levels (SGOT/SGPT) and liver biopsies bi-weekly for pathology over a period of several months.

PANELS AND STANDARDS

Several investigators provided panels known to contain hepatitis A

antigen or antibody, which served as controls and/or standards in the development of, and subsequently the day to day use of, various immunoassays for the detection of HAAg and anti-HA.

Antibody to Hepatitis A

Paired Sera--

Human--Dr. S. Krugman (New York University School of Medicine) provided 10 sets of paired sera collected from patients at Willowbrook Hospital who had experienced clinically diagnosed MS-1 type hepatitis (1). These sera had been titered for anti-HA by Dr. M. Hilleman (Merck Institute for Therapeutic Research) using marmoset antigen in an immune adherence hemagglutination assay (39).

--Dr. D. Gibson (Walter Reed Armed Forces Institute of Pathology) provided paired sera from an experimentally infected volunteer from the Joliet Prison hepatitis study (30).

Marmoset--Drs. F. Deinhardt and D. Peterson (Rush-Presbyterian-St. Luke's Hospital, Chicago, Illinois) provided 6 sets of paired marmoset sera collected prior to inoculation of GBG (31) hepatitis A antigen and during convalescence from the induced clinically apparent disease.

Chimpanzee--Dr. J. Moor-Jankowski (Laboratory of Experimental Medicine and Surgery in Primates, Sterling Forest, New York) provided several sets of paired sera collected from chimpanzees during the course of experimental induction of clinical hepatitis A in these animals.

--Research Resources Branch of the National Institute of Allergy and Infectious Diseases provided paired sera which were distributed by this agency as standards.

Unpaired Sera--

ENLI Standards--We have obtained by plasmapheresis several units of convalescent plasma from a patient who developed hepatitis A during the epidemic at San Diego Naval Base (20). These units have been assayed by several laboratories, using various test procedures, and found to have a very high titer of anti-HA. This source has served as the major supply of gamma globulin used for IEM and RIA in these studies.

Individual small samples of human convalescent sera have been supplied by several investigators, including Dr. D. Bradley (Center for Disease Control, Phoenix, Arizona), Dr. W. Schultz (U.S. Naval Medical Research Institute) and Dr. W. McAleer (Merck Institute for Therapeutic Research).

Hepatitis A Antigen

Human--

Drs. D. Gibson and W. Bancroft (Walter Reed) provided fecal extracts

prepared from a Joliet volunteer experimentally given MS-1 antigen (30). These fecal samples had been studied by IEM for the presence of HAAg (17).

Marmoset--

Dr. W. McAleer (Merck Institute for Therapeutic Research) provided a small amount of marmoset liver HAAg (18).

The major source of antigen used to generate data from the assays reported in these pages came from stools collected at Lynchburg.

ASSAY PROCEDURES

Immune Electron Microscopy

Detection of Hepatitis A Antigen--

Paired sera, collected from a patient antecedent to and subsequent to hepatitis A infection were used as negative control and source of specific antibody to hepatitis A antigen (anti-HA). The assay was performed in the following way. To 0.9 ml of a 5% fecal suspension, 0.1 ml of a 1:10 or 1:100 dilution of these sera was added and the mixture was incubated overnight at room temperature. The suspension was centrifuged at 40,000 rpm in a Ti-50 rotor for 30 minutes and the resultant pellet resuspended in 0.1 ml deionized water. A drop of the suspension was placed on a 400 mesh carbon-coated grid, allowed to stand for two minutes and then drawn off. Thereafter the residue was fixed with 3% glutaraldehyde and stained with 3% phosphotungstic acid. The grids were examined in the electron microscope for the presence of HAV antigen-anti-HA antibody complexes. Five representative grid squares from each of three grids were examined before a suspension was judged positive or negative. Care must be taken to compare results of paired sera, since stool suspensions and sera contain a large variety of particulate matter.

Detection of Antibody to Hepatitis A--

The same procedure was followed as for the detection of HAAg, except that the fecal sample was a known positive and the serum (plasma) sample (diluted 1:100) was the unknown. The presence of HAV antigen-antibody complexes indicated that the serum contained anti-HA.

Radioimmunoassay

Detection of Hepatitis A Antigen--

The test is a solid phase "sandwich" type radioimmunoassay in which gamma globulin with high anti-HA activity is immobilized by adsorption to polyvinyl microtiter "U" plates (Cooke Engineering Co., Alexandria, Va.). The second element of the "sandwich" is the antigen contained in the test-sample and the third element is the ^{125}I-anti-HA gamma globulin, which is added after the sample has been removed from the reaction well. The non-reacted radioactive label is removed and the immobilized, reacted ^{125}I-anti-HA is then determined. Specifically, the test is prepared and performed in the following manner:

One hundred microliters (approximately 80 μg/ml) of chromatographi-

cally purified immunoglobulin from a serum with high anti-HA activity was placed in a series of microtiter wells. (A convalescent serum 1:1000 in PBS was also satisfactory.) The plate was covered with Parafilm and incubated at 4°C overnight in a moist chamber. Thereafter the IgG was removed and the wells rinsed with 0.01M PBS containing 2% fetal calf serum. The final rinse was left in the wells for 15-20 minutes. A 50 µl sample of the test materials (usually a 2-5% stool suspension) was added to each of two wells (all samples were run in duplicate) and incubated in a moist chamber at 45°C. Different sample-incubation times were used (2 hr, 4 hr, overnight), depending on convenience and level of sensitivity required. After the test samples were removed from the wells, the wells were washed (3X) with PBS. Then 75 µl of ^{125}I-labelled, chromatographically purified IgG (specific activity, 8-12 µCi/µg) containing high anti-HA activity was added to each well. The plates were incubated at 45°C for 2 hrs in a moist chamber, washed (5X) with PBS to remove unbound ^{125}I-antibody, the wells cut out and counted in a gamma counter for 1 min. Five samples known to be negative for HAV were run as negative controls. Any sample which was three times the mean radioactive counts per minute of these negative samples was considered reactive, that is, positive for HAV.

Detection of Antibody to Hepatitis A--

Direct Test--Sera to be tested for the presence of anti-HA were first diluted 1:1000 in 0.01M PBS. Then 100 µl of each serum was added to two microtiter wells and incubated overnight in a moist chamber at 25°C. The diluted antiserum was removed and the wells washed (3X) with PBS-FCS. In each well was placed 50 µl of a standard HAV-positive stool suspension. (The stool dilution factor was determined as that necessary to yield 2000-4000 cpm when assayed with a standard convalescent serum and with the ^{125}I-anti-HA used on the particular occasion.) The stool suspension was incubated for 2 hrs (or 18 hrs) at 45°C and washed (3X) with PBS. Then 75 µl ^{125}I-anti-HA (150,000 cpm) was added to each well and incubated for 2 hrs at 45°C. The radiolabelled antibody was removed, the wells washed (5X) with PBS, cut out and counted on a gamma counter for 1 min. Five wells coated with sera (1:1000) known to be nonreactive for anti-HA and 2 wells coated with the serum (1:1000) source of the ^{125}I-anti-HA were used as negative and positive controls. A serum specimen was considered to contain anti-HA if the ratio of its cpm to the mean cpm of the negative controls was greater than or equal to three.

Indirect Test--A "blocking test" was also used to detect the presence of anti-HA in serum/plasma specimens. For this test, the wells were not coated with the unknown serum, as in the direct assay, but with the standard anti-HA serum (1:1000). Coating of the wells and rinsing were done in the usual manner. Then 75 µl of a standard HAV-positive fecal suspension (approximately 2000 cpm) was added to the coated wells and incubated for 1 hr at 45°C. Prior to this addition, the fecal antigen had been incubated at room temperature for 15-20 min with an equal volume of the unknown serum (1:10 in PBS) in an uncoated well. This step permitted the "blocking" of the standard antigen if anti-HA was present in the unknown serum, with the subsequent reduction of radioactive cpm

from the incubation of 75 µl ^{125}I-anti-HA for 1 hr at 45°C. The positive and negative controls in this assay were known paired sera, collected before and after infection with hepatitis A. Antibody to hepatitis A was considered to be present in any serum sample which reduced the net cpm of the positive control by more than thirty percent. The net cpm of the positive control was determined as the cpm of the positive control minus the mean cpm of the negative control.

Mercaptoethanol-RIA-Test--This assay was developed in an effort to distinguish anti-HA (IgM) from anti-HA (IgG). The results are discussed in the appropriate section of the text. Sera were treated with mercaptoethanol under conditions known to convert the pentameric 19S IgM to the monomeric bivalent 7S IgM subunit. To 25 µl of 0.2M 2-mercaptoethanol ($HSCH_2$ CH_2OH) in PBS (0.15M NaCl, 0.01M KPO_4, pH 7.5) was added 25 µl of specimen serum. The mixture was incubated in a 37°C water bath for 2 hrs. Then 200 µl of PBS containing 0.03M iodoacetamide was added and incubated at 37°C for 15 min. With this solution as a 1:10 dilution of the original serum, a final 1:1000 dilution was made of each serum to be tested. Wells were coated in the standard fashion and the remaining steps of the assay carried out as described above for the "direct test."

Preparation of anti-HA IgG from Whole Serum--

Plasma known to contain high anti-HA activity was clotted by adding 2 units of thrombin per milliliter plasma. The mixture was incubated at 37°C for 1 hr and then kept at 4°C for 18 hrs. The clot was separated from the serum by centrifugation in a GSA rotor at 6000 rpm for 1 hr. The entire procedure was repeated on the recovered serum.

To purify the gamma globulin, the serum was first diluted (1:1) with cold distilled water. Then 28.2 g solid $(NH_4)_2SO_4$ was slowly added to 100 ml of diluted serum with constant stirring for approximately 1.5 hrs. To separate out the formed precipitate, the serum solution was centrifuged in a GSA rotor at 10,000 rpm for 60 min, the supernatant poured off and the pellet resuspended in 0.01M borate buffer (pH 8.0). A glass rod was used to resuspend the IgG to about 20% of the starting volume of serum. The dissolved precipitate was dialyzed for 18 hrs against 2 changes (6 liters each) of 0.01M KPO_4 (pH 7.5). The IgG solution was removed from the dialysis bag and clarified at 25,000 rpm for 30 min in a Spinco 30 rotor. The pelleted precipitate was discarded and the supernatant saved for coating microtiter wells for RIA and for further purification on a DEAE column to provide IgG for iodination with ^{125}I.

To prepare the chromatographic column, 100 to 150 g DE52 (Whatman, H. Reeve Angel, Pistcataway, N.J.) was washed 5 times with 4 liters of 0.1M KPO_4 (pH 7.5). The DEAE-cellulose was then washed 2X with deionized water and finally 6X with 0.01M KPO_4 (pH 7.5). The washed DEAE-cellulose was poured into a column 2.5 x 50 cm and then rewashed with 1500 ml of the same buffer.

The IgG enriched solution (described above) was applied to the column, which was then washed by 0.01M KPO_4 (pH 7.5) buffer. Fractions were

collected and all those with A_{280} >1.0 were combined.

Preparation of ^{125}I-IgG (anti-HA)--

IgG was purified from convalescent sera, known to have a high anti-HA activity, by the method of Sober and Peterson (40), as modified above, and stored between -20°C and -70°C at a concentration of approximately 8 mg/ml. Just prior to iodination, the IgG was diluted to 2.0 mg/ml with 1.0M KPO_4 (pH 7.5) and clarified by centrifugation at 10,000 x g for 5 min. The iodination procedure was essentially that of Hunter and Greenwood (41). 4.0 mCi (10-20 µl) of ^{125}I in 0.1N NaOH was added to 20 µl of Chloramine T (1.0 mg/ml in 1.0M KPO_4, pH 7.5). The reaction was continuously stirred in a conical microreaction vessel. 100 µl of IgG (2.0 mg/ml) was added and the reaction terminated after 15 sec by the addition of 50 µl of sodium metabisulfite (4.0 mg/ml) and 500 µl potassium iodide (10 mg/ml).

The iodinated IgG was separated from other components by Biogel Chromatography. The iodinated sample was applied to a Biogel A 0.5m column (1.5 cm x 95 cm) and eluted at a flow rate of 10 ml/hr. The elution buffer was 0.2% BSA, 0.1% NaN_3, 0.5% KI, 0.1M Tris-Cl (pH, 7.1). Fractions of 1.3 ml were collected from the column and the iodinated IgG was located by counting the radioactivity in 10 µl aliquots from the collected fractions.

Immune Adherence Hemagglutination Assay

Procedure--

The IAHA used was that described by Miller et al. (39). All test sera assayed were inactivated at 56°C for 30 min and the assay was performed using Cooke Microtiter "U" bottom plates. 50 µl of heat inactivated sample was placed in well #1 of duplicate microtiter lines and serially diluted out, using 25 µl blanks of gelatin veronal buffer (GVB) and Cooke tube manual diluters. Standard HAAg at appropriate dilution (4 IA units) was added to one line and GVB was added to the duplicate line as an anticomplement control. The plate was mixed on a Thomas vibro mixer (10 sec) and incubated at 37°C for 1 hr. Guinea pig complement (1:100) was added to all wells (25 µl), the plate shaken for 10 secs and incubated at 37°C for 40 min. Then 25 µl dithiothreitol (3 mg/ml) in 0.4M EDTA-GVB was added to each well, mixed for 10 sec and allowed to stand at room temperature for 3 hrs to allow a pattern of agglutination to develop. Only those wells with a 4+ agglutination were considered to be positive. A control antibody and antigen was assayed each time the test was run.

Preparation of Standard Antigen--

Using veal infusion broth adjusted to pH 8.5 with borate buffer containing 0.05% polyethylene oxide, a 5% fecal suspension was prepared from a Lynchburg stool specimen shown by IEM and RIA to contain a large amount of HAAg. The raw stool was weighed in a small plastic bag and the

appropriate volume of buffered broth added. It was then homogenized for 2-3 min in a "Stomacher 70" (provided by Cooke Laboratory Products, Alexandria, Va.). The suspension was centrifuged at 1000 x g for 10 min, the supernatant removed and recentrifuged at 10,000 x g for 10 min. The above process was repeated and the ensuing second extract kept separately as a source of HAAg. The fecal pellet was discarded and the supernatant from the first extract mixed (1:1) with 50% acid washed kaolin in 0.85% saline. The kaolin had been previously washed several times with distilled water until centrifugation at 1000 x g for 5 min left a clear supernatant above the kaolin pellet. The kaolin-stool suspension mixture was incubated in a 37°C water bath for 30 min with frequent vortexing. Then it was centrifuged at 2000 rpm in a GLC-2 for 5 min, the supernatant transferred to a clean tube and respun at 3000 rpm for 10 min. A dilution series of this antigen was run against an antibody control to determine the antigen dilution containing four IAHA units/25 μl.

Density Gradient Ultracentrifugation

Four to eight milliliters of a 5% stool suspension was layered onto a 30 ml CsCl linear gradient (from 1.15 gm/cm^3 to 1.50 gm/cm^3) in a buffer composed of 1.5% normal goat serum, 0.001M Tris (pH 7.2), 0.1% sodium azide and 0.4% bovine serum albumin. The gradients were centrifuged at 45,000 rpm for 16 hrs in a Spinco Ti-60 rotor and fractionated into 1 ml fractions using an ISCO Model D Density Gradient Fractionator. The gradient samples were dialyzed overnight against PBS (0.01M sodium phosphate, pH 7.2, and 0.15M NaCl) with one change of buffer. The fractions were assayed for hepatitis A by RIA and/or IEM and combined as desired.

Alternatively, HAAg in 10% fecal extracts was clarified by passage through a Sepharose 2B column prior to density gradient centrifugation. This was accomplished in the following way:

The fecal sample was first centrifuged at 20,000 rpm for 10 min in a Beckman Ti-50 rotor to remove all debris with a sedimentation coefficient greater than 1500S. Then 5.0 ml of the clarified supernatant was chromatographed on a 2 x 100 cm Sepharose 2B column at a flow rate of 6 ml/hr. Fractions of 5 ml each were collected and monitored for optical density at 280 nm and by RIA for the presence of HAAg.

SECTION 4

RESULTS

CHARACTERIZATION OF HEPATITIS A ANTIGEN

A total of 451 5% stool suspensions collected from 292 ambulatory patients resident in 8 wards at the Lynchburg Hospital and Training School during an outbreak of hepatitis A were examined by immune electron microscopy (IEM) and radioimmunoassay (RIA) for the presence of hepatitis A antigen. Of the 292 patients, 69 were diagnosed as having clinical disease (i.e., fever, malaise, diarrhea, dark urine, and jaundice; bilirubinemia and elevated serum glutamic pyruvic transaminase, SGPT), 54 as subclinical (SGPT weekly serum levels rose above 50 units) and 169 as asymptomatic (i.e., SGPT serum levels remained below 50 units). Asymptomatic patients were not necessarily considered to be uninfected. A summary of the source of the Lynchburg stool samples is given in Table 1.

TABLE 1. STOOL SAMPLE COLLECTION FROM LYNCHBURG TRAINING SCHOOL AND HOSPITAL

	Patients			
	Clinical	Subclinical	Asymptomatic	Total
Stool Samples Collected	90	91	270	451
No. of Patients	69	54	169	292
No. of Wards Studied (4A,4B,4C,5A,5C,29A,25A,25B)				8

Morphology and Serology

Thirty-eight (38) Lynchburg stool suspensions were shown to contain 27 nm electron dense virus-like particles which formed antigen-antibody complexes (Figure 1) in the presence of antibody found in convalescent sera which was not present in the corresponding acute or pre-bleed sera taken from the same source. This was true in sera from human patients naturally exposed to hepatitis A as well as those exposed experimentally; it was also true in paired sera from experimentally infected marmosets and chimpanzees. Morphologically, the particles were identical to those reported by others in published studies on hepatitis A. The serologic similarity of the Lynchburg 27 nm virus-like particles to those isolated

from other hepatitis A outbreaks is summarized in Table 2. In all cases, antibody elicited in the patient in response to the viral infection reacted with the HAAg recovered from each geographically separated source to form easily identified, characteristic complexes (Figure 1).

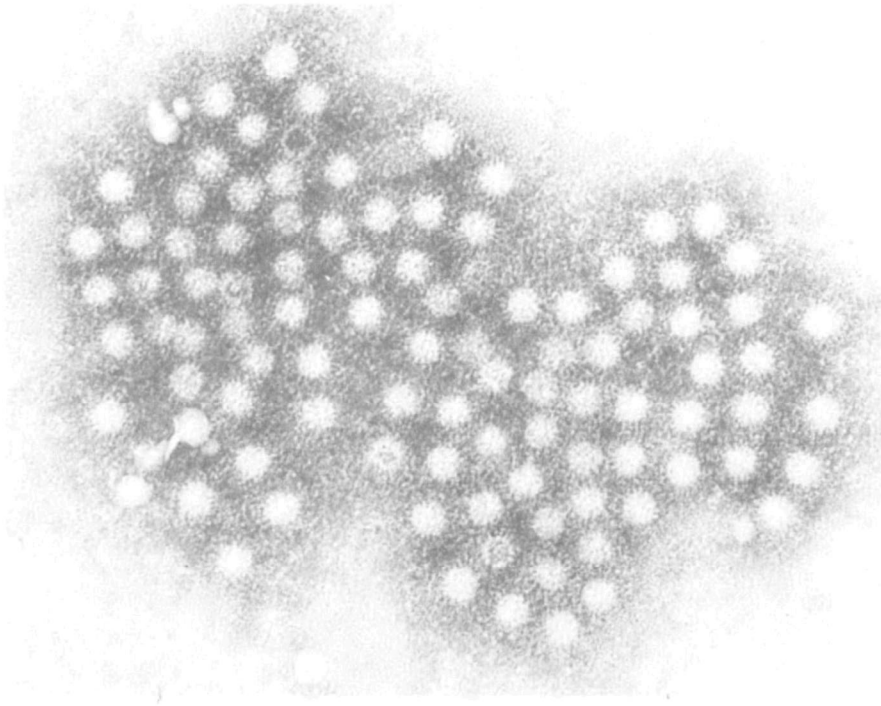

Figure 1. Electron micrograph showing an immune complex of 27 nm HAAg particles and anti-HA (X 150,000).

In addition to the 27 nm HAAg, a virus-like particle with a diameter of 23 nm was also found, sometimes in high concentrations (Figure 2). However, this particle did not show a consistent pattern by IEM of complexes (or non-complexes) based on the anticipated presence or absence of anti-HA in paired sera, and thus was not considered to be the hepatitis A virus.

Buoyant Density

The buoyant density of HAAg in human and marmoset stools was determined by isopycnic banding in preformed CsCl density gradients. IEM and RIA were performed on fractions of the gradients to detect the presence of the antigen.

Both human and marmoset feces known by RIA and IEM to have contained HAAg were subjected to CsCl gradients for buoyant density determinations of the contained virus.

TABLE 2. SEROLOGIC RELATIONSHIP OF 27 nm LYNCHBURG FECAL ANTIGEN TO PREVIOUSLY REPORTED HAAg FROM GEOGRAPHICALLY WIDESPREAD SOURCES

	2% Stool Suspension					
				Marmoset	MS-1 Joliet[c]	
Serum Samples	Lynchburg	San Diego[a]	Germany[b]	Liver	Pre (FJ1-2-4)	Acute (FJ-1-2-5)
Lynchburg (pre)	-	-	-	-	-	-
Lynchburg (conv)	+	+	+	+	-	+
Joliet (pre)[c]	-	-	NT	NT	-	-
Joliet (conv)[c]	+	+	NT	NT	-	+
Phoenix (pre)	NT	NT	NT	NT	NT	NT
Phoenix (conv)[d]	+	+	+	+	-	+
San Diego (pre)	NT	NT	NT	NT	NT	NT
San Diego (conv)[e]	+	+	+	+	-	+
Chimpanzee (pre)[f]	-	-	-	-	-	-
Chimpanzee (conv)[f]	+	+	+	+	-	+
Marmoset (pre)[g]	-	-	-	-	-	-
Marmoset (conv)[g]	+	+	+	+	-	+

pre = Specimen collected prior to transaminase elevation
conv = Specimen collected during convalescent stage
+ = Antibody-coated 27 nm particles seen by EM
- = Antibody-coated 27 nm particles not seen by EM
NT = Sample not tested

Reagents were kindly supplied by the following: (a) W. Schultz and J.A. Routenberg, (b) G. Fröesner, (c) D. Gibson, F. Top and W. Bancroft, (d) D. Bradley and J.E. Maynard, (e) W. Schultz, (f) Research Resources Branch (NIAID) and (g) F. Deinhardt and D. Peterson

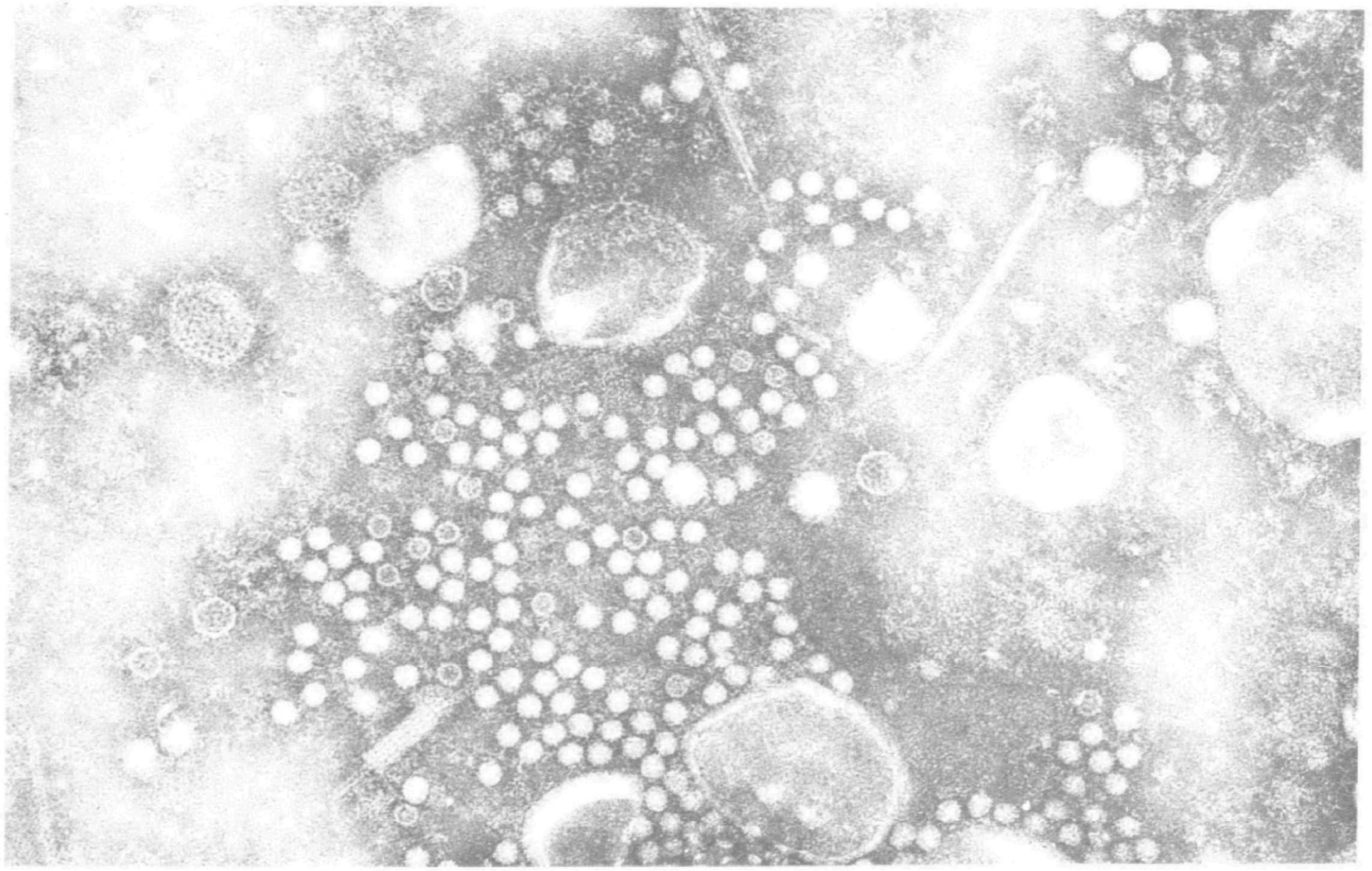

Figure 2. Electron micrograph of fecal extract containing large concentration of 23 nm virus particles (X 120,000).

The human stool extract was collected 7-10 days after peak SGPT elevation from an 8 year old female patient with sub-clinical hepatitis A. Fractions collected from the density gradient contained peaks of radioactivity at 1.27, 1.33 and 1.41 g/cm^3 (Figure 3). Each of these peaks contained 27 nm particles when examined by IEM. Figure 4 shows a complex of 27 nm particles banded in CsCl at 1.41 g/cm^3. The major peak of virus from the human stool was at 1.33 g/cm^3, while the only RIA peak of activity from the marmoset feces was at this same density (ρ = 1.33-1.35 g/cm^3).

The marmoset stool extracts were taken from the same animal at days 14, 22 and 35 post inoculum of GBG antigen. The animal developed signs of clinical hepatitis by day 36, as indicated by elevated SGPT and liver biopsy. RIA examination of fecal samples collected for 80 consecutive days post inoculum showed 3 periods of antigen excretion. Stools from the peak day of each of these periods were put on gradients for antigen density determination.

DISTRIBUTION OF HEPATITIS A ANTIGEN

Relation to Type of Hepatitis A

Of the 451 fecal samples examined, 38 were reactive by RIA for the presence of HAAg. Of these, 9 were from patients with clinical hepatitis, 25 from sub-clinical and 4 from asymptomatic. Although published data have reported the recovery of fecal HAAg only from stools collected prior to peak transaminase elevation, 12 Lynchburg stools collected at or after SGPT peak contained HAAg. The richest source of antigen was found in a stool collected from an 8 year old female with sub-clinical hepatitis approximately 10 days after maximum SGPT elevation. The frequency of anti-positive stools in relation to the type of hepatitis disease and level of SGPT is summarized in Table 3.

TABLE 3. FREQUENCY OF FECAL SAMPLE RIA-REACTIVE FOR THE PRESENCE OF HAAg RELATIVE TO TYPE OF DISEASE AND SGPT ELEVATION

	SGPT Elevation				
Disease State	Pre-Peak	Peak	Post-Peak	"Normal"	Total
Clinical	3	2	4		9
Sub-clinical	19	0	6		25
Asymptomatic				4	4

Virus Excretion Patterns in Experimental Animals (Marmosets)

Since the exact day of infection could not be determined in any of

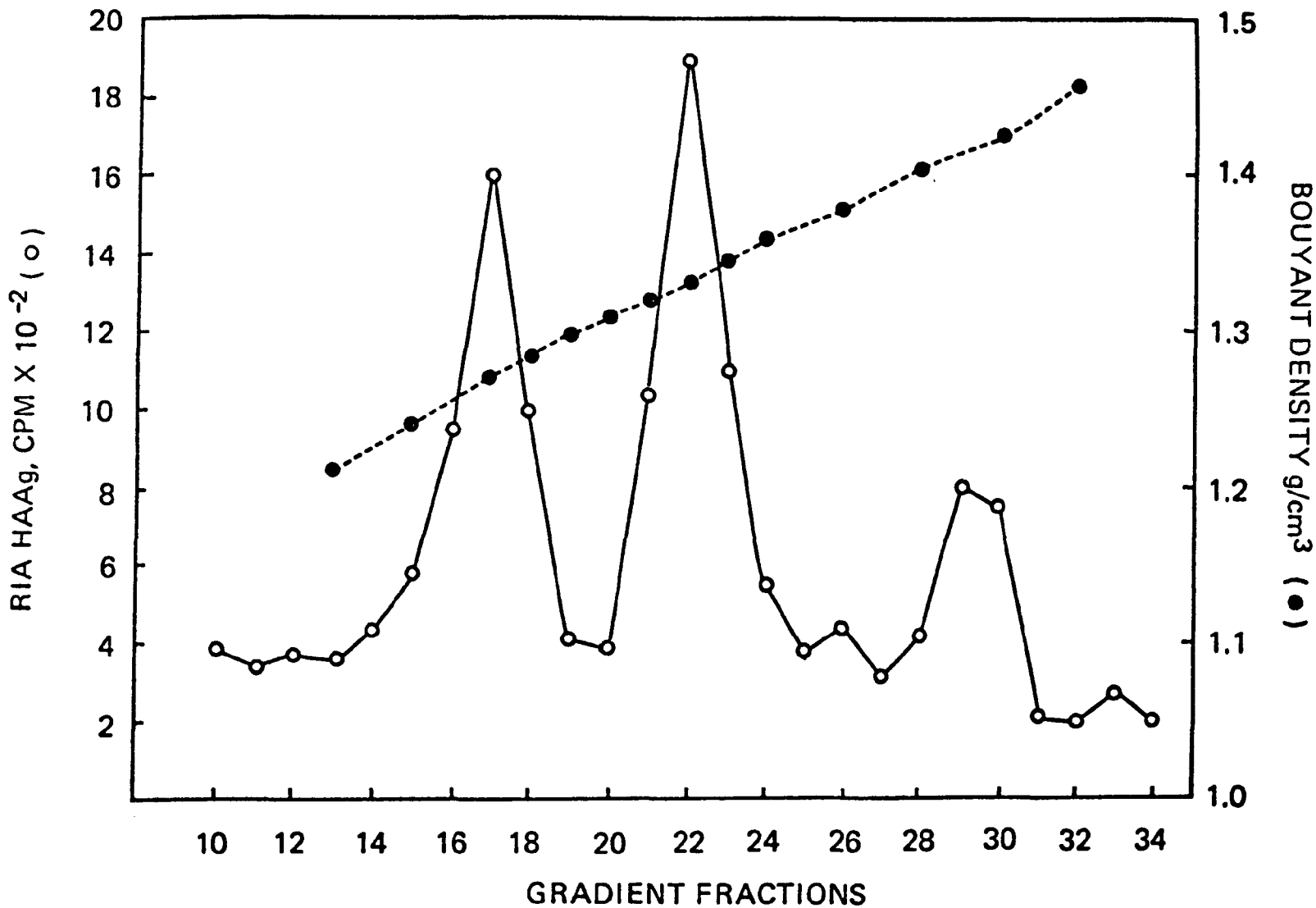

Figure 3. Isopycnic banding of a human post-acute phase fecal suspension in a CsCl density gradient.

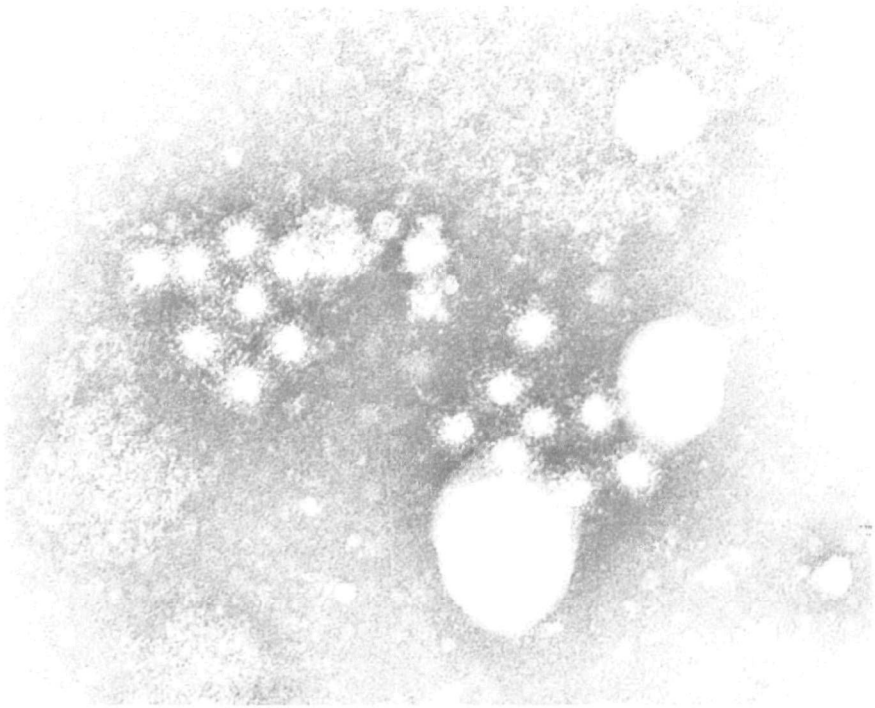

Figure 4. Electron micrograph of complexed 27 nm particles banded at 1.41 g/cm^3 in CsCl (human stool) (X 150,000).

the Lynchburg patients and the number of stool samples collected per patient was very limited, 6 marmosets were inoculated with HAAg to study the pattern of virus excretion. In this group, 4/6 marmosets developed acute hepatitis 29-43 days post inoculation. Clinical symptoms existed for 1-3 weeks. Depending upon the animal, HAAg was detectable in the feces by RIA from 8-48 days post inoculation and the excretion period of 10-20 days generally preceded maximal SGPT elevations by 6-21 days. A typical pattern of antigen excretion is shown in Figure 5.

Two animals which did not develop abnormal transaminase levels (or morphologic liver changes) had inapparent HAAg infections as demonstrated by antigen fecal excretion (5-19 days post inoculum) and development of anti-HA by day 36 post inoculum (Figure 6). Selected fecal specimens from the HAAg excretion periods in animals with acute or inapparent disease were examined by IEM and revealed typical 27 nm particles.

DISTRIBUTION OF ANTIBODY TO HEPATITIS A

It has long been recognized that mentally retarded patients living in institutions are highly susceptible to viral hepatitis. Attack rates of

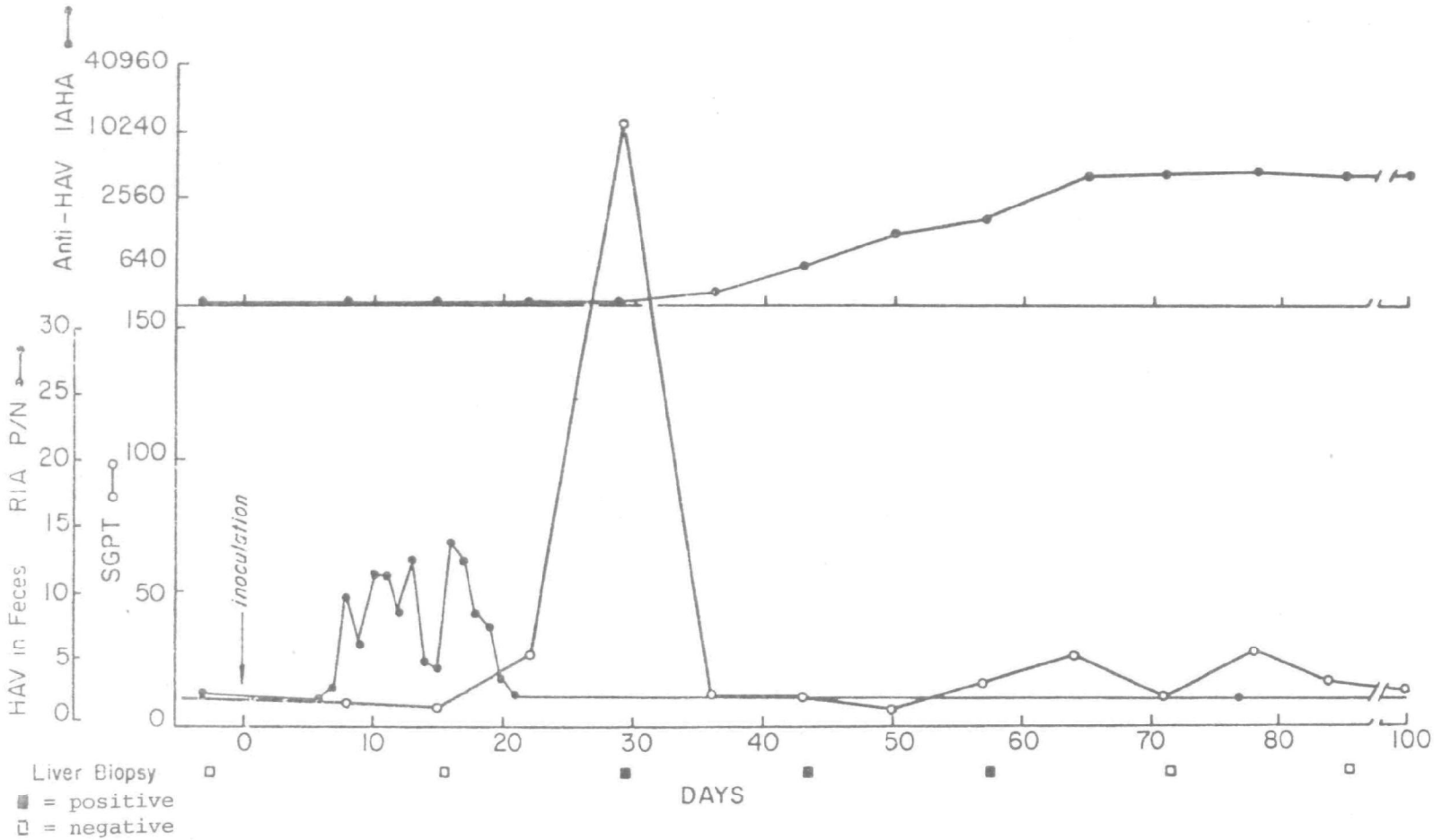

Figure 5. Typical pattern of HAAg excretion in a marmoset experimentally infected with hepatitis A (GBG) virus.

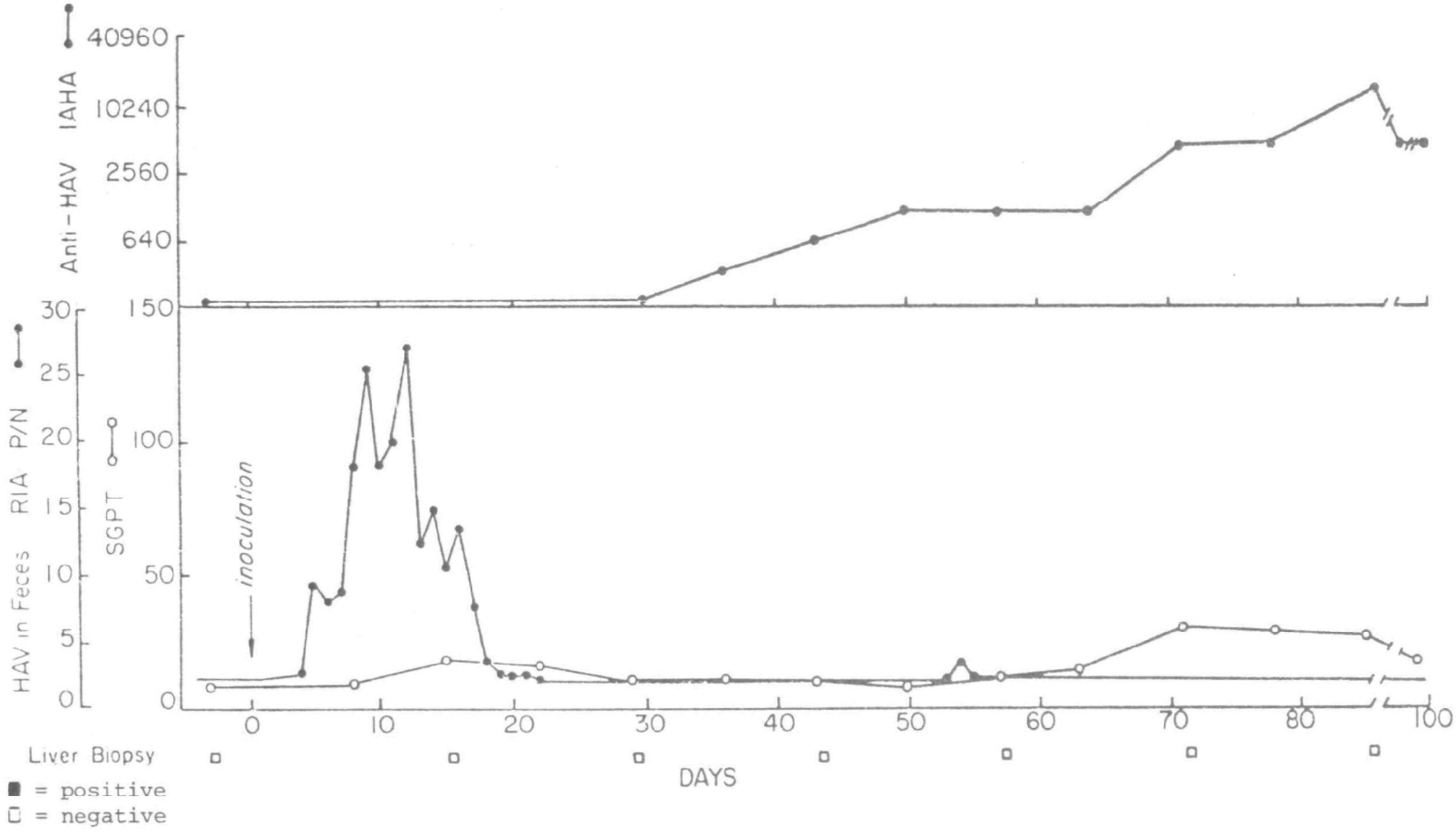

Figure 6. Inapparent hepatitis A in a marmoset experimentally infected with hepatitis A virus.

acute viral hepatitis during epidemics in such institutions have reached high levels. Approximately 16% (565/3600) of the entire institutionalized complement of Lynchburg Training School and Hospital and 57% (269/473) of the most severely retarded patients contracted clinical hepatitis in the epidemic described by Matthew et al. (29) and reported herein. With the discovery of a source of fecal HAAg (during the RIA screening of the Lynchburg stool samples), it became possible to develop both RIA and IAHA assays for the detection of antibody to hepatitis A and thus to obtain some insight into the level of exposure to this antigen experienced by the patients over the course of time and into the level of protection against reinfection offered by preexisting antibody.

Frequency of Anti-HA at Lynchburg

In Table 4 are summarized the results of RIA and IAHA tests for the presence of anti-HA among patients in the 10 wards housing the most severely mentally retarded. Of the 397 patients, sera were not available from 12. Thus the determinations were gathered from 385 patients. Of these, 41.6% (160) possessed anti-HA. The remaining 225 were without antibody and thus supposed to be at high risk. This assumption was borne out by the fact that 78.7% (177/225) contracted hepatitis A during the epidemic.

Hepatitis A has long been considered by epidemiologists (42) to be primarily a disease of children. An analysis of the distribution of anti-HA among Lynchburg patients according to age and sex supports this opinion. Only 5% (4/81) of the patients under 15 years of age had antibody to HAAg prior to the epidemic, while 34.3% (59/172) had anti-HA in the 15-29 year old age group and 72.4% (76/105) in the 30-49 year old group. Eighty-one (81) percent of the patients over 50 years of age had developed detectable levels of circulating antibody. These results appear in tabular form in Table 5.

Protective Effect of Natural Antibody (Active Immunization)

The existence of specific antibody to HAAg was previously assumed on the basis of apparent immunity to experimental reinfection (1, 43). Direct serological determinations made at Lynchburg established the factual basis of this assumption. As previously mentioned, 41.6% of the patients in the 10 wards studied had anti-HA prior to the hepatitis epidemic. None of this group showed any signs or symptoms of hepatitis A during the period of observation (1970-71), nor any clinical signs of hepatitis subsequently. This is in marked contrast to those patients at risk, i.e., lacking antibody: 77.9% (60/77) of those under 15 years of age, 91% (103/113) among the 15-29 year olds and 45% (13/29) among the 30-49 year olds developed hepatitis. All of this group now possess detectable circulating antibody to hepatitis A. These data are summarized in Table 6.

Protective Effect of Immune Serum Globulin (ISG)

During the first 10 weeks of the epidemic, commercial immune serum

TABLE 4. FREQUENCY OF ANTIBODY TO HEPATITIS A AND TYPE OF DISEASE AMONG PATIENTS IN TWO WARDS AT LYNCHBURG TRAINING SCHOOL AND HOSPITAL WHICH WERE EXPOSED TO HEPATITIS A ANTIGEN

Ward	No. of Patients	Age (Range)	Hepatitis A Disease State			Anti-HA(+)	$HB_SAg(+)$
			Clinical	Sub-clinical	Asymptomatic		
4A	36	13-28	14	7	15	14	8
4B	34	13-34	6	13	15	11	5
4C	46	15-35	12	10	24	20	4
5A	42	20-65	12	3	27	25	4
5B	51	19-63	1	1	49	38	4
5C	46	20-65	5	4	37	30	2
8B	56	6-20	45	7	4	3	2
25A	24	6-22	4	4	16	1	4
25B	24	6-22	12	7	5	4	5
29A	38	14-41	4	6	28	14	8
	397*		115	62	220	160	46

*Includes 12 for which data were unavailable

Hepatitis A Positive	177/385	(46%)
Anti-HA Positive	160/385	(41.6%)
Anti-HA Negative	225/385	(58.4%)

$$\frac{\text{Disease Pos.}}{\text{Anti-HA Neg.}} = \frac{177}{225} = 78.7\%$$

TABLE 5. FREQUENCY OF HEPATITIS A AND ANTIBODY TO HEPATITIS A ANTIGEN BY AGE AND SEX AMONG 385 PATIENTS AT LYNCHBURG TRAINING SCHOOL AND HOSPITAL

Age (Yrs)	Patients Tested		Anti-HA (+)		Hepatitis A Disease	
	Males	Females	Males	Females	Males	Females
0-4	1	0	0	0	1	0
5-14	5	76	0	4 (5.3%)	(80%) 4	56 (73.7%)
15-29	124	48	(36.3%) 45	14 (29.2%)	(58.9%) 73	30 (62.5%)
30-49	97	8	(72.2%) 70	6 (75%)	(13.4%) 13	0
≥50	26	0	(80.8%) 21	0	0	0
Total	253	132	(53.8%) 136	24 (18.2%)	(40%) 91	86 (65.2%)

TABLE 6. PROTECTIVE EFFECT OF ANTIBODY TO HEPATITIS A EXISTING PRIOR TO THE 1970-71 EPIDEMIC AT LYNCHBURG

Age (Yrs)	Patients Anti-HA(+)	Patients Disease(+)	Patients Anti-HA(-)	Patients Disease(+)
0-4	0	0	1	1
5-14	4	0	77	60
15-29	59	0	113	103
30-49	76	0	29	13
50	21	0	5	0
Totals	160	0	225	177

globulin (ISG) was widely administered without apparent protective effect (29). Therefore, a controlled study was undertaken the better to evaluate the effectiveness of ISG.

At the end of the study indications were that ISG prophylaxis was effective, if given at the appropriate time. However the distribution of preexisting antibody in the test population, the level of protection afforded by this antibody and the number of patients actually at risk was unknown. With the development of in vitro diagnostic techniques (described in this report) it was possible to reexamine the sera collected from patients in the two wards used for the controlled experiment.

In these wards, ISG was given six and eight days respectively prior to the appearance of the first clinical case of hepatitis A. The members of the wards - all ambulatory patients - were divided alphabetically into three groups: one received no ISG; a second received a dose of 0.02 ml/lb body weight and a third received 0.08 ml/lb. In the latter two groups a maximum of 10 ml ISG was given. The dose was repeated thirty days later. Serum samples were collected prior to initial ISG administration (pre-bleed) and 2, 3, 4, 5, 6, 8, 10, 12, 14 and 17 weeks thereafter. They have since been collected annually, where possible.

The two wards were composed of 60 patients; 23 received no ISG, 21 received 0.02 ml/lb body weight and 16 received 0.08 ml/lb. In the first group - those receiving no ISG - 19 of the 23 patients were shown to be at risk. Of these, 12 developed either clinical (8 patients) or sub-clinical (4 patients) hepatitis. On the other hand, 4 of 25 patients at risk in the two groups receiving ISG developed hepatitis, of which three cases were sub-clinical. Two of these four had their SGPT elevation on the very day ISG was given, one seven days post and the fourth fourteen days after inoculation. This would suggest that all four were infected prior

to the administration of ISG. A summary of the results is shown in Table 7.

Comparative Sensitivity of Assays to Detect Anti-HA

As described in Materials and Methods, several assays exist for the detection of antibody to hepatitis A. They include RIA (44, 45), IEM (17) and IAHA (39). From published data (17, 39, 46, 47), it was apparent that all of these assays were capable of detecting circulating antibody present in plasma collected during convalescence from the disease. The more important information from a diagnostic point of view was the comparative sensitivity of the various assays in detecting early antibody, that is, the IgM arising immediately after exposure to HAAg (48). To this end, two populations were studied by three assay methods.

In the first experiment, 10 serum samples from each of 15 Lynchburg patients who had experienced clinical hepatitis A were assayed by IEM, RIA and IAHA for the presence of anti-HA. The first serum sample was collected in each case at least 3 weeks before peak SGPT elevation and the last sample in each series was collected approximately 2 years after peak SGPT. Table 8 contains the data from one of these patients and is essentially the same as the other 14 series. The times are presented as weeks before and after peak transaminase levels were reached. The RIA (P/N) ratio was determined as the quotient of the cpm of each serum sample divided by the cpm of the first sample of the respective series. A P/N value ≥ 3.0 was considered to be positive. In every series, a positive RIA and IEM assay indicating the presence of anti-HA was obtained in sera collected at SGPT peak elevation, and generally by one week prior to the peak. IAHA titers were not positive until 3-4 weeks later. RIA and IAHA results on serial serum samples of five patients are summarized in Table 9 to demonstrate the greater sensitivity of RIA in detecting early antibody.

Course of Development of Anti-HA

Because the actual day of exposure to HAAg could not be determined with certainty among the patients at Lynchburg, an experiment was performed on six adult white-lipped marmosets to obtain more precise data on the course of development of anti-HA (a description of the procedures is detailed in Materials and Methods).

As in the human study, the IEM and RIA assays detected the presence of antibody prior to SGPT elevation, while the IAHA required at least an additional two weeks - a time when the SGPT level was returning to near normal. Detectable levels of circulating antibody were present in the marmosets within 28 days after the injection of HAV. Table 10 shows a comparison of IAHA, IEM and RIA titers of anti-HA in a typical series of marmoset serum samples.

Both the human and marmoset serial serum samples were treated with mercaptoethanol at a concentration sufficient to disrupt IgM. This approach was used in an effort to develop a system to distinguish IgM from

TABLE 7. EFFECTIVENESS OF IMMUNE SERUM GLOBULIN IN PREVENTING HEPATITIS A INFECTION AS RELATED TO NUMBER OF PATIENTS AT RISK

ISG Dose ml/lb	No. in Group	No. with Hepatitis Clinical	Subclinical	Total	No. of patients without HAAb	% of HAAb(-) patients infected	
0.0	23	8	4	12	19	63%	(12/19)
0.02	21	1	3	4	15	26.6%	(4/15)
0.08	16	0	0	0	10	0%	(0/10)

A Chi-square (1 degree of freedom) test was applied to the attack rates of those with and those without ISG.

$X^2 = 11.183$ $P = <0.005$

TABLE 8. COMPARISON OF IAHA, IEM AND RIA TITERS OF ANTI-HAAg IN HUMAN SERIAL SERUM SAMPLES

Sample No.	Date	SGPT[a]	IAHA[b]	IEM[c]	RIA (cpm)	P/N[d]
2012	8/10/70	18	<20	0	80	1.0
2723	8/26/70	25	<20	1-2+	242	3.0
3179	9/2/70	2800	<20	2-3+	1156	14.4
3913	9/9/70	330	<20	2-3+	1091	13.6
4163	9/18/70	90	<20	2-3+	1112	13.9
4243	9/23/70	37	<20	3-4+	1074	13.4
4580	10/8/70	15	1600	3-4+	1282	16.0
4618	11/3/70	18	1600	3-4+	1220	15.2
5348	12/9/70	15	3200	3-4+	1186	14.8
6346	11/6/72	18	3200	3-4+	1202	15.0

(a) SGPT expressed in International units.
(b) IAHA titer expressed as reciprocal of final dilution giving 4+ agglutination.
(c) IEM expressed as relative amount of antibody complexed to antigen particles.
(d) P/N = ratio of cpm of particular serum divided by the cpm of the first sample in the series.

TABLE 9. COMPARISON OF RIA AND IAHA TITERS OF ANTIBODY TO HAAg IN HUMAN SERA.

	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5	
Date	RIA[a]	IAHA[b]	RIA	IAHA	RIA	IAHA	RIA	IAHA	RIA	IAHA
8/10/70	133	<20	136	<20	145	<20	159	<20	80	<20
8/28	126	<20	688	<20	669	<20	148	<20	242	<20
9/2	127	<20	1009[c]	<20	863[c]	<20	697	<20	1156[c]	<20
9/9	754[c]	<20	1132	<20	626	<20	830[c]	<20	1091	<20
9/18	1320	<20	762	<20	793	<20	956	<20	1112	<20
9/23	740	<20	906	400	835	<20	935	<20	1074	<20
10/8	915	400	1154	3200	948	1600	1161	800	1282	1600
11/3	1514	3200	1565	6400	ND	3200	2140	6400	904	1600
2/10/71	1805	6400	1519	≥12800	1456	≥12800	1505	6400	980	1600

(a) RIA values expressed as counts per minute.

(b) IAHA titer expressed as reciprocal of final dilution given 4+ agglutination.

(c) SGPT peak.

TABLE 10. COMPARISON OF IAHA, IEM AND RIA TITERS OF ANTI-HAAg IN MARMOSET SERIAL SERUM SAMPLES

Sample No.	Date	SGPT	IAHA	IEM	RIA(cpm)	P/N
C9581	2/3/76	12	<20	-	844	1.0
HAAg Inoculum	3/24/76		-	-	-	-
C7493	4/1/76	12	<20	-	823	1.0
C9008	4/8/76	8	<20	-	812	1.0
C4087	4/15/76	12	<20	±	1276	1.5
C3956	4/22/76	30	<20	+	2838	3.4
C1332	4/29/76	148	<20	+	3805	4.6
C2661	5/6/76	18	640	+	5708	6.8
C0608	5/13/76	10	640	+	5867	7.0
C5571	5/20/76	26	1280	+	5662	6.8
C3873	5/27/76	52	2560	+	5777	6.9
C6287	6/3/76	55	5120	+	6185	7.4
C1402	6/10/76	50	5120	+	7453	8.9

IgG antibody response. In theory, the mercaptoethanol would inactivate the IgM and thus reduce the radioactivity of the serum samples collected during the early stages of the disease, at a time when the IgM-anti-HA would be present in greater concentration than IgG-anti-HA. Figure 7 shows that the actual result was opposite from expected. The mercaptoethanol enhanced the determination of the IgM response, probably by rupturing the pentameric 19s IgM to its monomeric moieties, thus providing more antigenic sites for attachment of the ^{125}I-anti-HA.

Figure 7 also serves to summarize the studies on marmosets experimentally infected with HAAg. In general, HAV excretion occurred before SGPT elevation, though, in this case, antigen was still found in the stools at the time of highest transaminase levels. Circulating anti-HA detectable by RIA was already present in the marmoset by the time abnormal SGPT levels were recorded. Measurable IAHA anti-HA titers lagged behind RIA titers by several days and early RIA titers (thought to be of the IgM class) were rendered more detectable by mercaptoethanol treatment.

Frequency of Anti-HA in General Population

During the course of this research grant, two reports appeared on the variation found from lot to lot in several commercial sources of immune serum globulin (39, 49). Data from several authors suggested that about 47% of the adult population of the United States have anti-HA (34, 50, 51, 52) thus at the same time being immune to further hepatitis infection and potentially an excellent source of ISG for hepatitis prophylaxis. Therefore, we determined the frequency of anti-HA in a commercial plasma donor population and the titer (IAHA) of circulating antibody.

Antibody to hepatitis A antigen at an IAHA titer of 1:10 or greater was detected in 91 of 245 (37%) regular plasma donors tested. Ninety of these positive samples were also reactive at a dilution of 1:100. Table 11 contains a summary of the frequency of anti-HA in this population according to age, sex and race. There was a statistically significant ($P < 0.01$) increase in the frequency of anti-HA between the 18-29 year old group (30%) and the 30-49 year old group (57%). A similar statistically significant difference was found among Lynchburg patients for these respective age groups (34% vs. 72%). There was no significant difference between the frequency of anti-HA in whites and blacks nor in the frequency of anti-HA between the white sexes. There was not a sufficient number of black females in the donor population for statistical comparison.

The frequency of anti-HA in this population according to IAHA titer is summarized in Table 12. Approximately 32% (29/91) of the positive donors, or 12% (29/245) of all the donors had an anti-HA titer of at least 1:1000. While the 30-40 year old group contained only 21% (51/245) of the donor population tested, 48% (14/29) of the high titered plasma donors were in this group.

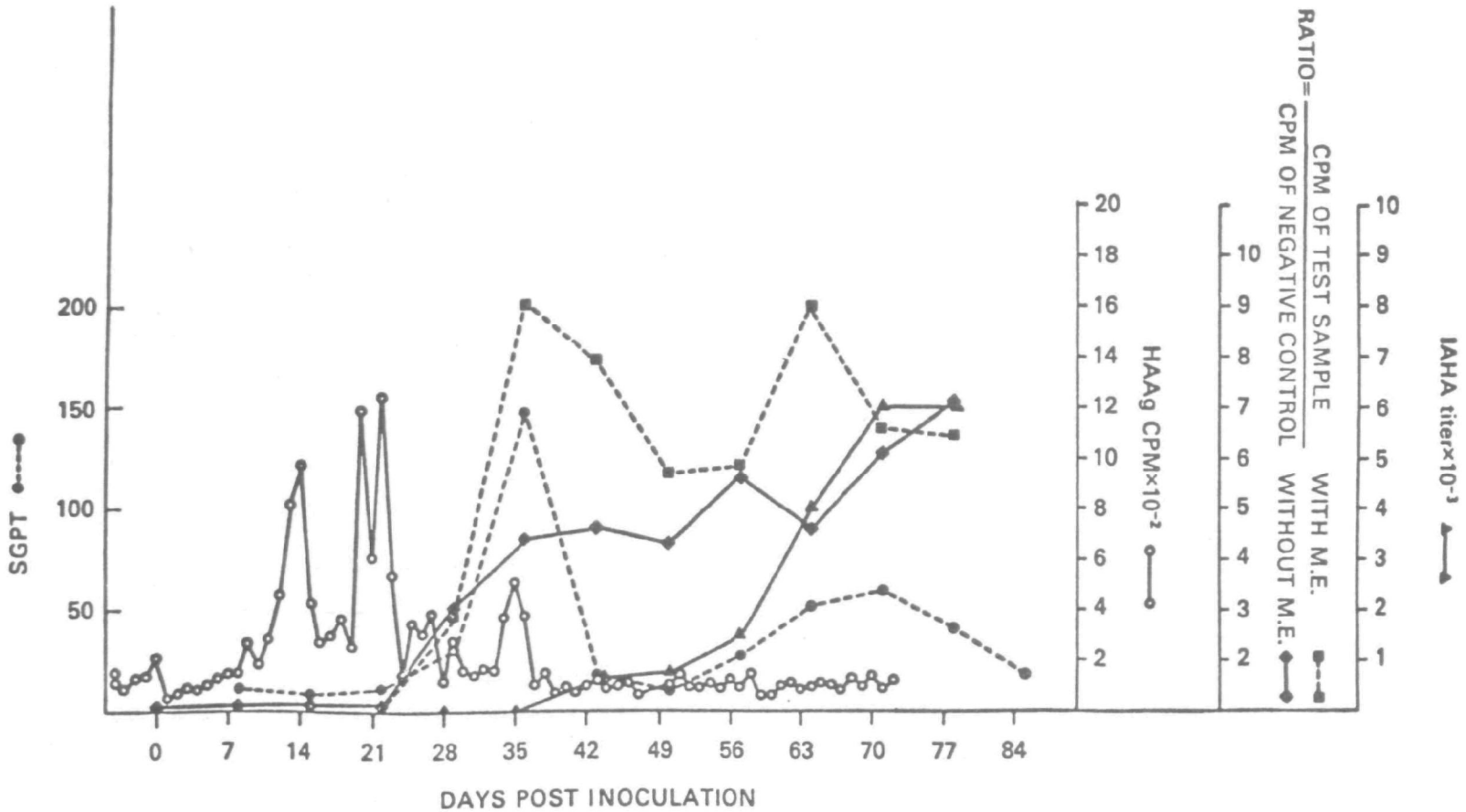

Figure 7. Pattern of HAAg excretion, SGPT levels and anti-HA development during the course of clinical hepatitis A in an experimentally infected marmoset.

TABLE 11. FREQUENCY OF ANTIBODY TO HEPATITIS A ANTIGEN AMONG A GROUP OF REGULAR PLASMA DONORS AT PLASMA ALLIANCE ACCORDING TO AGE, RACE AND SEX

Age (Yrs)	White				Black				Total	
	Male		Female		Male		Female			
18-29	27/89*	(30)	18/68	(26)	11/29	(38)	1/1	(100)	57/187	(30)
30-49	13/21	(62)	14/22	(64)	1/5	(20)	1/3	(33)	29/51	(57)
≥50	4/6	(67)	1/1	(100)	0/0	(0)	0/0	(0)	5/7	(71)
Total	44/116	(38)	33/91	(36)	12/34	(35)	2/4	(50)	91/245	(37)

* $\frac{\text{No. of donors positive for anti-HA}}{\text{Total number of donors}}$

( ) Percent of donors positive for anti-HA for respective group.

TABLE 12. FREQUENCY OF ANTIBODY TO HEPATITIS A ANTIGEN ACCORDING TO AGE AND TITER AMONG A GROUP OF PLASMA DONORS AT PLASMA ALLIANCE

	Reciprocal of IAHA Titer						
Age	10	100	1000	2000	3000	6000	Total
18-29	1*	41	7	1	5	2	57
30-49		15	8	2	3	1	29
≥50		5					5
Total	1	61	15	3	8	3	91

* No. of samples positive for anti-HA at the indicated dilution, but negative at the next higher dilution.

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16. ABSTRACT This research program was initiated with the overall objective of combining the techniques of electron microscopy, ultracentrifugation, column chromatography, tissue culture and serology to identify, isolate and characterize the etiologic agent of infectious hepatitis, to propagate it in cell cultures and to develop *in vitro* immunodiagnostic assays capable of detecting the presence of the hepatitis A virus and its antibody both in the patient and the environment.

Through the program, it was possible morphologically and serologically to relate this agent to those isolated from other geographically separated hepatitis A epidemics. Employing hepatitis A antigen detected by RIA screening and isolated from stool specimens collected from patients with both clinical and subclinical hepatitis, a radioimmunoassay and an immune adherence hemagglutination assay were developed for the detection of antibody to hepatitis A. Using these assays, the protective effect of circulating anti-HA against reinfection and the prophylactic effect of commercial ISC containing anti-HA were established. Likewise, a survey of a commercial plasma donor population was conducted to obtain information on the distribution of anti-HA among the population as a whole. It was found that approximately 37% of the tested donors had anti-HA with the frequency varying directly with age.

All attempts to propagate the hepatitis A virus in tissue culture have been unsuccessful.

17. KEY WORDS AND DOCUMENT ANALYSIS

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