Studies of Plasma Protein Synthesis by Peripheral Cells from Normal Persons and Patients with Infectious Mononucleosis

By Arch A. MacKinney, Jr.

Infectious mononucleosis has been described as having all the properties of leukemia except that it is self limited. Thus, it interests the investigator as a model for the malignant lymphoid diseases more than as a mild clinical illness of adolescence and early adulthood. It is a generalized infiltrative disease of the lymphoid organ, characterized by atypical lymphocytes in the peripheral blood and by an unusual macroglobulin, the heterophile antibody. Morphologic and serologic changes are well known. We have tried to find out what these changes mean in terms of cell biology.

We previously showed that atypical lymphocytes from patients with infectious mononucleosis included a population which was actively dividing. Five per cent of the cells were in DNA synthesis, fifty fold more than were present in normal peripheral blood. Cells in metaphase were demonstrated after 1 hr. incubation in vitro and the rate of division was constant for 5 hr. thereafter.

The appearance of infectious mononucleosis cells suggests that they may also be participating in an immunologic process. Previous cytochemical studies of infectious mononucleosis cells showed either no evidence of globulin synthesis or immunofluorescence in rare plasmacytoid cells. Recently, van Furth and co-workers found gamma globulins synthesized by infectious mononucleosis cells in tissue culture by isotopic labeling. In the studies reported here, we have quantified the amount of radioactive protein synthesized in normal and infectious mononucleosis cultures and tested the specificity of globulin synthesis by examining for newly formed heterophile antibody. The data indicate that these cells synthesize several proteins including heterophile antibody, an unusual macroglobulin.

METHODS

Blood was drawn from patients hospitalized in the Student Infirmary of the University of Wisconsin Hospitals. Patients were selected when the presumptive diagnosis of infectious mononucleosis was made on clinical grounds and when more than 10 per cent atypical lymphocytes were present. All but one patient had diagnostic titers of heterophile antibody.

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body. Patients who were convalescent were rejected. Sixteen patients were studied 7 to 28 days after onset of symptoms. Seven normal individuals provided control material.

Heparinized autologous plasma and leukocytes were obtained by spontaneous sedimentation of red blood cells at 37 C. for 30 to 60 min. Neutrophils were not separated from lymphocytes. Leukocytes fresh from peripheral blood were examined for heterophile-antibody production by the mixed agglutination method of Zaalberg,7 using washed infectious mononucleosis cells and sheep red cells. The agar-plate technic of Jerne et al.8 was applied using ox red cells in place of sheep red cells to determine the presence of hemolytic antibody.9

The majority of procedures were performed on supernatants from tissue culture. The tissue-culture procedure was designed to give maximum radioactivity in protein and optimal cell proliferation with minimal contamination from cell breakdown products.

Two ml. of plasma and cells and 8 ml. of tissue culture medium 199 were combined with 0.12 ml. of Phytohemagglutinin P (Difco; lot 457925) and leucine 14C 1 μc. (New England Nuclear, S.A. 275 mc./m.mol.) per ml. of culture fluid. The final cell concentration was 1000 to 3000/mm³. Cultures were incubated 68 to 168 hr. at 37 C. At the end of the incubation, the cells were removed by centrifuging at 2000 g. for 30 min. The supernatant fluid was tested against sheep erythrocytes. The titer was compared to serum titer at time zero of incubation. The culture fluid was dialyzed in the cold against normal saline for three days to remove free leucine 14C, and concentrated by perevaporation or dialysis against Carbowax (20M) in buffered saline for 8 hr. It was then re-equilibrated by dialysis against saline overnight. Aliquots were stored frozen at -10 C.

The culture fluid was examined for total trichloracetic acid (TCA) precipitable radioactivity as follows: One-tenth ml. samples in duplicate were precipitated with an excess of 10 per cent TCA. After 12 hr. in the cold, the precipitates were washed three times with ethanolether 1:1, once with ether and incubating in vacuo overnight. The precipitates were dissolved in 1 ml. hydroxide of Hyamine or 0.05 ml. 1 N NaOH and counted in a liquid scintillation counter, using dimethyl POPOP 100 mg./L. toluene at an efficiency of approximately 70 per cent determined by external standardization. In some experiments, 10 ml. of tissue culture supernate were precipitated with TCA. Paired 4 mg. samples of dry TCA protein were weighed and counted to determine specific activity.

Tissue-culture supernates were analyzed by routine immunoelectrophoresis on agar slides and autoradiographed using Tri-X film for four to six weeks. Rabbit anti-whole human serum was used in the trough.

Three normal and three infectious mono culture supernates were fractionated using C-200 Sephadex. One ml. of dialyzed, concentrated fluid was passed through a 2.5 × 45 cm. Sephadex column and eluted with 0.1 M tris HCl in 0.2 M NaCl, pH 8.0 at 4 C. The flow rate was 10 ml./hr. and fifty to sixty 2 ml. samples were collected. The distribution of immunoglobulins IgG, IgA, and IgM was determined by double diffusion in agar against specific antisera. Fractions were assayed for protein content by light absorption at 280 μ and for radioactivity by liquid scintillation counting of 0.1 ml. samples in Bray’s mixture.10 The protein peaks were tested for heterophile antibody activity using washed sheep erythrocytes.

Other cultured supernatant aliquots were precipitated in tubes with anti-IgM, anti-IgG, and anti-albumin. One-tenth ml. samples were incubated with antisera (antibody excess) for 1 hr. at 37 C., then 48 hours at 4 C. After centrifugation at 4,500 rpm for 20 min., the samples were washed in cold saline until the supernatant was free of counts. Duplicate precipitates were dissolved in 1 N NaOH and assayed for total protein at 280 μ or for radioactivity in the liquid scintillation counter.

Thirteen infectious mono culture sera and twelve normal culture materials were reacted with sheep infectious mono receptor generously provided by Dr. Georg Springer.11 This material is a potent, partially purified heterophile antigen extracted from sheep erythrocytes which forms a precipitate with heterophile antibody. It was diluted with saline-phosphate buffer (1/15 M pH 7.2) to a final concentration of 0.5 mg./ml. One-tenth ml. of
Fig. 1.—Agar-slide immunoelectrophoresis of two infectious mono culture supernates (A, C) and their autoradiographs (B, D). Albumin is on the left, migrating toward the anode. IgG is on the right. Both autoradiographs show labeled IgG and albumin lines, and a prominent prealbumin line which is unidentified. D shows probable radioactive IgA line.

culture fluid and 0.15 ml. of heterophile antigen were incubated at 37 C. for 1 hr. and for three to six days in the cold. The precipitates were washed three times with cold saline, dissolved in 1 N NaOH, and counted in the liquid scintillation counter with Bray's mixture. Addition of antigen or antibody to supernatant did not bring down further radioactivity. Saline-phosphate buffer was substituted for antigen as a blank for each study, since small shreds of precipitated protein appeared in all samples. The blank was subtracted from the test sample to give net counts. Sera preincubated at room temperature for 1 hr. in 0.1 M mercaptoethanol (0.1 ml. serum and 0.1 ml. 0.2 M ME.) served as additional controls. In 7 of the 12 experiments with normal tissue culture supernates, nonradioactive infectious mononucleosis serum was added to bring the heterophile antibody titer into the pathologic range. In each study, net dpm were divided by dpm in TCA-precipitable protein of a similarly prepared sample, to give per cent radioactive protein.

Results

Estimation of antibody synthesis by whole cells using Zaalberg and Jerne technics showed no difference between normal and infectious mononucleosis cells. These results agree with others using whole cell technics.4,5

TCA-precipitable protein contained three times as much radioactivity in PHA-stimulated cultures as in unstimulated controls in both normal and infec-
Fig. 2.—G-200 Sephadex fractionation of infectious mononucleosis tissue culture supernate. Note first sharp peak of high-molecular-weight radioactivity compared to the small amount of protein present. Heterophile activity and IgM were found in this peak, as well as some IgA.

No difference in the specific activity of total protein (dpm/mg. TCA protein) was found between culture supernates from infectious mononucleosis cells and normals. Cultures incubated with all constituents except cells showed no TCA-precipitable radioactivity. Heterophile antibody titer did not increase after culture, a not unexpected finding in view of the small number of cells cultured. There was no apparent correlation between duration of illness or proportion of atypical lymphocytes and the amount of radioactivity in protein.

Radioactive arcs of IgG, probably IgA, albumins, and other unknown proteins were seen on autoradiographs of immunoelectrophoresis plates. $^{14}$Carbon-labeled IgM lines were not seen. Much of the radioactivity appeared in nonspecific smudges apparently composed of lipoproteins. There was no difference between normal and infectious mononucleosis studies. Figure 1 illustrates immunoelectrophoresis patterns and their autoradiographs.

G-200 fractionation of serum proteins is based on molecular size and shape. The protein fraction containing IgM was eluted first, followed by a second peak containing IgA and IgG, with albumin-sized molecules trailing. This study showed high specific activity IgM (DPM/OD) in addition to the other radioactive globulins seen on electrophoresis. The IgM peak contained the heterophile antibody. Figure 2 shows fraction of an infectious mononucleosis culture supernate.
PLASMA PROTEIN SYNTHESIS

Table 1.—Estimates of Newly Synthesized Serum Proteins from Antiglobulin Precipitation

<table>
<thead>
<tr>
<th>dpm/0.1 ml. Culture Fluid</th>
<th>Specific Activity (dpm/od) of precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>Inf. mono. mean</td>
<td>90</td>
</tr>
<tr>
<td>Range</td>
<td>38-211</td>
</tr>
<tr>
<td>No. tested</td>
<td>6</td>
</tr>
<tr>
<td>Normal mean</td>
<td>274</td>
</tr>
<tr>
<td>No. tested</td>
<td>6</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

Table 2.—Heterophile Antigen-Antibody Reactions Per Cent Radioactivity Precipitated

<table>
<thead>
<tr>
<th>Sera</th>
<th>Mean %</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C inf. mono.</td>
<td>4.4</td>
<td>0.0-19.5</td>
<td>13</td>
</tr>
<tr>
<td>14C normal</td>
<td>0.16</td>
<td>0.0-0.6</td>
<td>5</td>
</tr>
<tr>
<td>14C norm. + I.M.</td>
<td>0.20</td>
<td>0.0-0.9</td>
<td>7</td>
</tr>
</tbody>
</table>

*dpm in heterophile antigen-antibody precipitate divided by dpm in total protein precipitated with 10 per cent TCA. Sera were tissue culture supernates from thirteen patients with infectious mono and normal subjects. Nonradioactive serum with high titer of heterophile was added to seven normal sera to enhance coprecipitation. The amount of antibody precipitated in infectious mono sera is significantly greater than in the pooled normal studies; p = <0.01

Cultures supernates precipitated with antiglobulin antisera showed radioactivity in all three classes of immunoglobulin as well as albumin. The rank of radioactivity in serum proteins was albumin > IgG > IgA > IgM. The specific activity of the proteins (DPM/OD) was reversed: IgM > IgA > IgG > albumin. Normal cultures had significantly larger amounts of radioactivity in albumin than infectious mononucleosis cultures. Table 1 summarizes the data.

In experiments using infectious mononucleosis supernate from thirteen patients, 4 per cent of TCA-precipitable radioactivity was precipitated by heterophile antigen. The range was 0 to 19 per cent. The radioactivity precipitated by the antigen was reduced 25 to 75 per cent by prior incubation with 0.1 M mercaptoethanol, suggesting that the radioactivity was associated with macroglobulin. The radioactivity in cultured fluid from five normal experiments was less than 1 per cent. In seven normal precipitin test samples to which high titer heterophile antibody carrier was added, no radioactivity in excess of 1 per cent was observed. Since the two groups of experiments on supernates from normal cultures appeared homogenous, they were pooled for comparison with the infectious mononucleosis test series. The higher radioactivity in infectious mononucleosis cultures was significant with a p value of less than 0.01 (Table 2).
Cells in peripheral blood of patients with infectious mononucleosis make heterophile antibody as shown by a succession of findings. Incubation of cells with leucine $^{14}$C resulted in radioactivity in acid-washed proteins. This suggests synthesis of protein rather than nonspecific binding. Radioactivity was found in macroglobulins by both gel filtration and immunoprecipitation. Radioactivity was precipitated from infectious mononucleosis culture supernates but not normal supernates in the presence of heterophile antigen. Addition of "cold" heterophile antibody to normal culture supernates did not result in significant increase in radioactive precipitate. The precipitin reaction was mercaptoethanol-sensitive as predicted for a macroglobulin antibody.

The atypical lymphocyte is an interesting prospect for this and other IgM synthesis. Previous workers have localized macroglobulin synthesis to large and medium lymphocytes as well as lymphoid reticulum cells of human lymph nodes. Since atypical lymphocytes appear quite different from normal cells, the otherwise similar amounts of globulin synthesized was not expected. We suspect that phytohemagglutinin "drives" normal and infectious mono cell lines toward a common pathway. Inman and Cooper compared the ultrastructure of the PHA-stimulated normal cell and the atypical lymphocyte and found them very similar. Probably only specific antibody differences will be detected under these conditions of culture. Tao showed that PHA can provoke anamnestic immune globulin responses in vitro. Other mitotic stimulants may produce different patterns of protein synthesis. This question is the subject of current study.

Evidence from different laboratories and various technics points to immunoglobulin synthesis and release by peripheral blood cells in vitro. The amount of radioactivity is proportional to the number of lymphocytes cultured. Synthesis of protein is inhibited by puromycin. Radioactivity appears in predicted peptide spots after digestion with trypsin. Numerous independent methods of protein separation including salting out, immunoelectrophoresis, immunoprecipitation, and gel filtration confirm the synthesis of globulin. In our studies, the use of the latter two technics demonstrate highest specific activity in IgM.

Radioactivity has been found in a variety of proteins not thought to be synthesized by leukocytes. Albumins and unidentified immunoelectrophoresis arcs were labeled in our studies. Albumin probably binds some leucine $^{14}$C. Some unidentified lines may be globulin fragments. These unexpected findings will require further study.

A small number of studies have shown specific antibody synthesis by peripheral blood cells. Forbes found antithyroglobulin in one patient. Hulliger and Sorkin and Landy et al. showed antibody production with peripheral leukocytes from hyperimmunized animal cells in vitro. Our studies add heterophile antibody to the list.

The significance of atypical morphology of lymphocytes is not yet clear. Both IgM synthesis and proportion of cells in DNA synthesis appear to be

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increased early in the course of infectious mononucleosis, but Epstein and Brecher's study did not show good correlation between morphology and DNA synthesis. Our study does not suggest a correlation between the proportion of atypical lymphocytes in the sample and the amount of heterophile antibody synthesized.

We conclude that infectious mononucleosis cells apparently synthesize heterophile antibody. This, and active cell division already described, are two biochemical parameters which distinguish infectious mononucleosis cells from normal leukocytes.

**Summary**

Tissue culture of peripheral blood leukocytes with $^{14}$C leucine resulted in radioactive proteins identified as immunoglobulins by immunoelectrophoresis, gel filtration, and immunoprecipitation. Radioactive heterophile antibody was detected by precipitin reaction with heterophile antigen. It was found in significant concentration in infectious mononucleosis cultures but not in normal controls. Apparent synthesis of heterophile antibody and active cell division are two parameters which distinguish infectious mononucleosis cells from normals in vitro.

**SUMMARIO IN INTERLINGUA**

Ilistoculturas de leucocytos de sanguine peripheric con leumcina a $^{14}$C resultava in le formation de proteinas radioactive que esseva identificate como immunoglobuhinas per immunoelectrophorese, filtration a gel, e immunoprecipitation. Radioactive anticorpore heterophile esseva detegite per reaction precipitinic con antigeno heterophile. Illo esseva trovate a nivellos significative de concentration in culturas de mononucleosis infectiose sed non imi controlos normal. Le apparente synthese de anticorpore heterophile e un active division de cellulas es duo parametros le quales distingue cellulas de mononucleosis infectiose ab cellulas normal sub conditiones de cuturation in vitro.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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