action we absorbed the HL-A7 antiserum with β-lipoprotein from different HL-A7-positive and HL-A7-negative individuals under the previously determined optimal conditions. The absorbed serums were tested and some of the results obtained with two HL-A7-positive as well as two HL-A7-negative donors are shown as an illustrative example in Table 2. Absorption with the β-lipoprotein fraction of serum from donor R.C., who is HL-A7-positive, removed the cytotoxic antibody reactive with HL-A7-positive cells R.C., J.B., and J. B-J. The β-lipoprotein from HL-A7-positive donor J.B. completely absorbed the antibody activity for test cells R.C. and J. B-J. and reduced the antibody activity for cell J.B., indicating that there is some quantitative relationship between the amount of antigen present on the cells and in the plasma of a particular donor, which may vary from one individual to another. Absorption with the β-lipoprotein from HL-A7-negative donors, F.C. and N.D., failed to remove antibody activity. As expected, the HL-A7-negative cell, F.C., did not react with any of the absorbed serums.

These data furnish convincing evidence that there is a soluble HL-A7 antigen associated with the β-lipoprotein fraction of serum from HL-A7-positive donors. This is equatable with the human red cell isoantigens which have soluble counterparts. However, two distinct categories of soluble antigens exist in the erythrocyte systems.

In the ABO blood-group system the soluble antigens are secreted independently of the antigenic structures on the erythrocyte surface. In the Lewis system, on the other hand, the determinants are primarily soluble plasma polysaccharides and are secondarily adsorbed onto the red cell surface, thereby imparting the observed cellular polymorphisms (*5*).

The latter mechanism is intriguing and may gain further support from the findings of Morton *et al.*, who claim to have found the HL-A7 antigen on red cells (*6*). This was shown by means of a highly sensitive hemagglutination method which used the autoanalyzer and could be interpreted as meaning that the soluble HL-A7 lipoprotein was adsorbed onto the erythrocyte surface. Such a possibility is especially likely, since in their experiments washed, packed erythrocytes failed to absorb the antibody.

Although lipoproteins can be polymorphic (*7*), at our present state of understanding we do not know whether the β-lipoprotein antigen we are detecting is primarily soluble material which is adsorbed onto the surface of leukocytes or a degradation product resulting from normal cellular destruction.

The method of absorption devised for this study is well suited to antiserum purification. Large quantities of antiserums can be absorbed by enlarging the size of the column and increasing the amounts of antigen-containing serum and thus the amount of β-lipoprotein on the column. Batch procedures may also be employed.

Since the initiation of this work, van Rood *et al.* have reported that the antibody activity of HL-A2 and 7^b antiserums can be neutralized with whole serum from donors carrying these HL-A antigens (*8, 9*). Van Rood has also been able to sensitize skin graft recipients by injecting plasma from donors positive for the 7^b antigen (*8*). The use of purified β-lipoprotein fractions rather than lymphocytes could conceivably facilitate production of HL-A antibody.

These findings have additional implications. It is known that the transfusion of whole blood can stimulate the production of leukocyte antibodies. These can in turn cause hyperacute rejection of renal transplants, as pointed out by Kissmeyer-Nielson and his associates (*10*). Therefore, in order to avoid immunization of potential transplant recipients who are on chronic hemodialysis a method of rendering blood buffy-coat-free should be selected which also removes a large proportion of plasma.

RONALD K. CHARLTON*
CHESTER M. ZMIJEWSKI†
Division of Immunology,
Duke University Medical Center,
Durham, North Carolina 27706

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* Present address: Dental Research Center, University of North Carolina, Chapel Hill.
† Present address: Division of Transplantation Immunology, Ortho Research Foundation, Raritan, N.J. 08869.

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Tuberculin-Active Carbohydrate That Induces Inhibition of Macrophage Migration but not Lymphocyte Transformation

Abstract. *A tuberculin carbohydrate fraction, GAE, in sensitized animals induced a delayed type of skin reactivity and inhibited the migration of macrophages but failed to stimulate lymphocyte transformation in vitro. Tuberculin protein-containing fractions were active in each test. These results show that in vitro lymphocyte transformation is not necessarily a corollary of delayed type hypersensitivity.*

The ability of polysaccharides to elicit a delayed type of hypersensitivity has been a subject of controversy for a number of years. We have shown previously that GAE, a carbohydrate preparation from culture filtrates of *Mycobacterium bovis* (strain BCG) containing less than 0.4 percent nitrogen (in the form of peptide or protein) is skin reactive in tuberculin-sensitive guinea pigs and causes in vitro inhibition of migration of macrophages from sensitized guinea pigs (*1–4*). The present study shows that this carbohydrate had little or no lymphocyte-transforming activity even though it caused macrophage inhibition and had dermal reactivity in sensitized animals, whereas protein-containing fractions from the same culture filtrates were active in each test.

The antigens used were GA, GAE, GB, and GX (*1, 3–5*). These substances were prepared from the acid-soluble (*p*H 4.0) portion (G) of exhaustively dialyzed 3-month-old culture filtrates

Table 1. In vitro and in vivo activities of BCG fractions. Nitrogen values were Kjeldahl determinations. Carbohydrate values were calculated as glucose. Test values are averages of results obtained with four guinea pigs, ± 1 standard deviation. S/C = counts per minute of antigen-stimulated cells/counts per minute of control nonstimulated cells. Skin test values are the product of two orthogonal diameters of erythema. N.D., not done.

Fraction	GB	GA	GAE	GX
Nitrogen (%)	7.6	1.8	0.4	0.0
Carbohydrate (%)	51	100	104	104
	Sensitized animals			
Transformation (S/C)	5.5 ± 2.1	3.1 ± 0.7	1.5 ± 0.5	1.2 ± 0.4
Macrophage inhibition (%)				
Direct test	38 ± 7.4	38 ± 8.7	48 ± 7.9	99 ± 16.3
Indirect test	34 ± 4.1	31 ± 3.8	51 ± 3.1	98 ± 7.6
Skin test (5 μg) mm²	290 ± 34	198 ± 35	183 ± 35	0
	Unsensitized animals			
Transformation (S/C)	1.06 ± 0.42	0.73 ± 0.08	0.71 ± 0.15	N.D.
Macrophage inhibition (%)				
Direct test	98 ± 7	99 ± 7	97 ± 10	N.D.
Skin test (5 μg)	0	0	0	0

of BCG. Fractions GA and GB were separated from G by gel filtration through a Sephadex G-50 column. Fraction GAE was derived from GA by digestion with proteolytic enzymes, and GX was obtained as a gelatinous pellet by ultracentrifugation of G at 104,000*g* for 3 hours. Repeated washing of the pellet with distilled water removed all detectable nitrogen.

Male guinea pigs, Hartley strain, weighing about 400 g were sensitized by the injection of a total of 5 mg (dry weight) of heat-killed BCG in water-in-oil emulsions into the four paws. Two weeks after sensitization, cells from these animals were used for a concomitant study of lymphocyte transformation and macrophage migration inhibition. To demonstrate the production of transformation and migration inhibitory factor (MIF), lymphocytes from draining lymph nodes were cultured at 37°C with and without 25 μg of antigen at a concentration of 1.2×10^7 per 4.0 ml of medium RPMI 1640 (*6*) containing 20 percent fetal calf serum. The medium was replaced at 24 hours and the old medium examined for MIF. After 72 hours, 2 μc of tritiated thymidine was added to each culture and these cultures were further incubated overnight. The cells were washed and then digested with hydroxide of hyamine. The quantity of ^3H in the digests was counted by a liquid scintillation technique. Because quenching was slight and uniform, results were recorded in counts per minute (count/min) and expressed as the S/C ratio (counts per minute of antigen-stimulated cells/counts per minute of control nonstimulated cells). An S/C ratio of 1.0 indicates that no transformation above control unstimulated cells occurred and a ratio of 2.0 is considered to be significant (*7*).

For the direct MIF test (*8*), macrophages were withdrawn from the peritoneal cavity of sensitized guinea pigs 3 days after the injection of 30 ml of sterile mineral oil. The cells were washed with Hanks balanced salt solution, packed into capillary tubes, and the tubes were cut at the fluid-cell interface. The packed cell portion was placed in a chamber to which was added 12.5 μg of antigen, or no antigen, in RPMI 1640 medium with 10 percent fetal calf and 5 percent guinea pig serum. Chambers were incubated at 37°C and the "outgrowth" was projected, traced, and measured with a planimeter. Results were expressed as

$$\text{Migration index (MI)} = \frac{\text{Outgrowth with antigen}}{\text{Outgrowth without antigen}} \times 100$$

The 24-hour cell culture fluids were assayed for MIF (*9*), after purification by elution through Sephadex G-75 columns. Unsensitized guinea pig peritoneal exudates were used as the indicator cell population for the indirect MIF assay.

Intradermal skin tests with 5, 1.25, and 0.31 μg of antigen per 0.1 ml were done in guinea pigs 2 weeks after sensitization. Skin reactions were read at 24 hours and recorded as the product of two diameters of erythema. All values shown are averages of the reactions obtained with four animals.

The activities of the fractions as measured by lymphocyte transformation, by macrophage migration inhibition, and by skin reactions are compared in Table 1. In sensitized animals, lymphocyte transformation induced by the fractions was directly related to N content: GB (7.6 percent N) was the most reactive, GA (1.8 percent N) was less reactive, and GAE (0.4 percent N) and GX (0.07 percent N) had no significant activity. On the other hand, macrophage migration inhibition and delayed type of dermal activity were not correlated with N content. Fractions GB and GA caused significant inhibition and to the same degree, while GAE was only slightly less active and GX was inactive. Fraction GB had the greatest dermal activity, whereas GAE and GA of equal activity were strong reactors but somewhat less so than GB; GX was inactive. None of the fractions induced reactions in cells or skin of unsensitized animals.

Failure of in vitro transformation has also been noted with pneumococcal polysaccharides by Oppenheim *et al.* (*10*) and with a different tuberculin polysaccharide by Heilman and McFarland (*11*). These investigators could not demonstrate delayed skin reactivity with their carbohydrate fractions, while our polysaccharide GAE was almost as skin reactive as tuberculin (PPD) (*1–4*). Our findings are in agreement with those of Gerety *et al.* (*12*) who have shown that pneumococcal polysaccharide SSS II can induce skin reactivity in sensitized guinea pigs and cause the inhibition of macrophage migration. Unfortunately, they did not investigate the transforming potential of their polysaccharide. The importance of macrophages in the induction of transformation of sensitive lymphocytes by antigen has been reported (*13*). However, we question whether macrophages are necessary for the production of MIF. It would be of interest to know if carbohydrates such as GAE and pneumococcal polysaccharides can be adequately processed by macrophages in vitro. If not, this could account for the negative transformation observations reported here.

Carbohydrate GX is devoid of demonstrable protein and has been shown previously (*1*) and in these studies to be unable to induce a delayed skin reaction or to cause inhibition of migration of macrophages. Only one out of six rabbits injected with GX produced precipitating antibodies. Thus different preparations of bacterial carbohydrates may vary in their immunogenicity. The small quantity of protein in GAE may be important in providing polarity for interaction with the antigen-sensitive cells responsible for an immune response. This interaction may trigger a response analogous to that described by Paul *et al.* (*14*). They demonstrated that type III pneumococcal polysaccharide caused antigen-

sensitive cells to produce antibody without significant replication. Likewise, GAE must have stimulated some of the pathways, but not necessarily those involved in cellular replication. Further, Bennett and Bloom (*15*) have observed that transformation of lymphocytes was not a requirement for MIF production. In their studies MIF appeared in supernatant cultures within 6 hours after antigen stimulation, but morphologic alterations of lymphocytes were not seen prior to 24 hours. Godfrey *et al.* (*1*) demonstrated inhibition of macrophage migration as early as 2 hours. It is suggestive that mediators, such as MIF, which result from the interaction of antigens with sensitized lymphocytes are produced either as a continuum of sequential events along the same pathway that culminates in blast transformation and mitosis or by pathways separate from those that lead to mitosis. Although the pathway of mediation was not elucidated, our results showed that polysaccharide fraction GAE stimulated the sensitive committed lymphocyte to the point of MIF production which correlated with delayed skin reactivity but not to the point of DNA synthesis or cellular proliferation.

SOTIROS D. CHAPARAS
DANIEL E. THOR
H. P. GODFREY
HAROLD BAER, SALLY R. HEDRICK
Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

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Localization of 5S RNA Genes on Drosophila Chromosomes by RNA-DNA Hybridization

Abstract. *[^{3}H]RNA with a high specific activity was prepared from larvae of* Drosophila melanogaster *grown 4 days in contact with [^{3}H]uridine. Purified tritiated 5S RNA was annealed to the DNA of polytene chromosomes, which had been denatured in formamide. The 5S RNA genes are placed within the region 56E-F of the right arm of chromosome 2. This localization was determined from autoradiographs, where the radioactivity from hybrids of [^{3}H]RNA and DNA was confined to the 56E-F segment.*

The ribosome from prokaryotic and eukaryotic organisms contains three RNA species, one molecule each of 23 to 28*S*, 16 to 18*S*, and 5*S* RNA, as well as numerous proteins. The 5*S* RNA is associated with the 23 to 28*S* RNA in the larger ribosomal subunit. The haploid *Drosophila melanogaster* (*1*) genome has between 130 to 190 copies each of the genes coding for the 18*S* and 28*S* RNA. These are found at the nucleolus organizer sites on the X and Y chromosomes. Tartof and Perry (*1*) have recently concluded that in *D. melanogaster* the haploid genome contains about 200 5*S* genes, roughly a number equal to the number of either the 18*S* or 28*S* genes. Furthermore, they excluded the sex chromosomes as the site of the 5*S* genes. An earlier investigation by Brown and Weber (*2*) in the toad, *Xenopus laevis*, showed that the 5*S* genes are not interspersed with the 18*S* and 28*S* genes and that there are 20,000 to 27,000 copies per haploid genome. This redundancy is 45 to 60 times that of the 18*S* and 28*S* genes and is in marked contrast to the situation in *Drosophila*. However, there is agreement in the two studies in that the site of the 5*S* genes is placed outside of the nucleolus organizer. In this study we have located the 5*S* genes cytologically in the genome of *D. melanogaster* from autoradiographs produced after hybridizing tritiated 5*S* RNA to denatured DNA of polytene chromosome squashes in situ.

First instar larvae (about 0.1 g) were placed on 10 ml of low yeast medium (*3*) containing 10 mc of [5-^{3}H]uridine (25 c/mmole, New England Nuclear Corp.). After 4 days' growth the third instar larvae were harvested by being floated in 60 percent sucrose (*4*). The RNA was extracted from the larvae by means of a modification of the phenol method in which sodium dodecyl sulfate was used (*5*). Column chromatography on methylated albumin Keiselguhr (MAK) (*6*) was employed to separate the RNA species. The fractions containing the 4*S* and 5*S* RNA species from the MAK column were dialyzed against a solution of 0.9*M* NaCl and 0.09*M* sodium citrate, at *p*H 7.4, in preparation for hybridization (*7*) and frozen until used. The radioactivity of the RNA taken from the MAK column was measured in a liquid scintillation spectrometer (*8*). The RNA had a specific activity of about 4×10^5 count min^{-1} μg^{-1} as measured on membrane filters where the counting efficiency was approximately 10 percent (4×10^6 disintegration min^{-1} μg^{-1}).

Squashes of salivary gland chromosomes from late third instar larvae were prepared and treated with ribonuclease (*7*). The DNA of the chromosomes was denatured by a 2.5-hour treatment in 95 percent formamide in 0.015*M* NaCl and 0.0015*M* sodium citrate (*p*H 7.4)

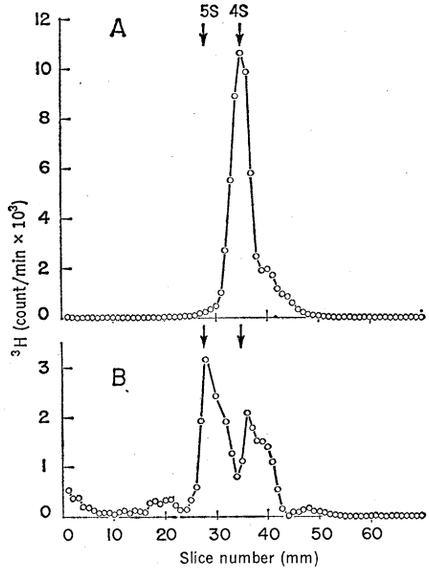

Fig. 1. Separation of tritiated 4*S* and 5*S* RNA on polyacrylamide gels (*9*). (A) The radioactivity profile of [^{3}H]RNA from the 4*S* peak from a MAK column. This peak of radioactivity coincides with the optical density maximum of unlabeled commercial transfer RNA (4*S*) from yeast (see arrow). (B) The radioactivity profile of the 5*S* RNA fraction taken from a MAK column. The main radioactivity peak agrees with the optical density maximum of 5*S* RNA taken from ribosomes from lily pollen (see arrow in A).