detected in human serum containing a high titer of herpes simplex antibodies if the serum was first precipitated with warm acetone. The test appeared to be applicable to blood or tissue level studies of any antiviral agent that shows significant *in vitro* activity.

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Isolation of Immunologically Competent Lymphocytes from Sensitized Mouse Spleens.* (28835)

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Large numbers of lymphocytes may be obtained from mouse spleens for immunologic studies. Although a number of methods have been reported for isolation of lymphocytes from blood and serosal exudates, these have not been satisfactory when applied to suspensions of splenic cells. This report is concerned with a simple method for preparation of highly purified suspensions of lymphocytes from splenic tissue. The red blood cells are removed by lysis and the lymphocytes are separated from other nucleated cells by centrifugation. Lymphocytes obtained by this procedure maintain their viability and immunologic competence.

Methods and results. Lymphocyte suspensions. Mice were sacrificed by cervical dislocation; the spleens were removed aseptically and minced immediately in a Petri dish at room temperature. Minced tissues from 2 to 3 spleens were suspended in 5 ml of tissue culture medium 199 and disrupted further by pipetting. This suspension of splenic cells contained many lymphocytes, as well as other nucleated cells and a variable number of erythrocytes. The suspension was filtered through 2 layers of lint-free fine gauze to remove the residual particles of tissue.

The suspension was centrifuged and the supernatant was discarded. Ten milliliters of unbuffered 0.35% saline was added to the packed cells and the cells were resuspended; this resulted in lysis of the erythrocytes. After one minute 1.2 ml of 5% saline was added to restore physiologic tonicity. The above procedure was repeated. The cell suspension, consisting of nucleated cells, was washed 3 times by resuspension in medium 199 and centrifugation. The supernatant was discarded and the cell mass placed into 12 cm segments of untreated polyethylene tubing (1.2 mm internal diameter) with a Pasteur pipette. One end of the tubing was closed by tying it into a knot with a small hemostat. The plastic tubes, tied-ends down, were put into open segments of glass tubing (12.0 × 0.8 cm) for support and placed into centrifuge cups. The tubes were centrifuged at 275 *g* for 10 minutes. The lower one-third of the packed cells in the plastic tubing, containing lymphocytes with an admixture of other nucleated cells, was cut off and discarded. The upper two-thirds of the cell pack consisted almost entirely of lymphocytes. An average of 96% (range 90-99%) of the cells were small or medium lymphocytes. The remaining cells were macrophages and plasma cells. Myeloid cells were rare in these preparations. An average yield of 40 million lymphocytes was obtained from each spleen.

Viability. The viability of the lymphocytes was established by a dye-exclusion test(1) as well as by motility studies. A drop of

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0.5% eosin in saline was added to a drop of the lymphocyte suspension, placed on a slide, covered by a coverslip and examined. About 99% of the cells were viable as demonstrated by the exclusion of the dye from their cytoplasm. Purified suspensions of lymphocytes in medium 199 with 2% inactivated fetal calf serum were placed into Rose chambers and studied at 36°C by time-lapse microcinematography with phase contrast optics. Unpurified preparations of splenic cells suspended in the same medium were used as controls. The cells in both the purified and crude preparations showed a similar degree of motility.

Immunologic competence. The immunologic competence of lymphocytes was determined as follows: BALB/c mice were sensitized to homologous L cells of C_3H mouse origin as described previously(2). The spleens were removed aseptically and cut into halves. One-half of each was used for preparation of a purified lymphocyte suspension as described above and the other for a crude suspension of lymphoid cells. Both the purified and crude suspensions of sensitized lymphocytes were adjusted to concentrations of 2 million nucleated cells/ml. The spleens of nonsensitized control BALB/c mice were treated similarly. Twenty-four hours earlier, 1 ml aliquots of a suspension of L cells (100,000) in 10% bovine serum and 90% medium 199 had been placed into Leighton tubes and incubated at 37°C. For each experimental group 6 tubes without coverslips for cell counts and 2 with coverslips for morphologic studies were used. The suspensions of lymphocytes were added to these cultures. After 48 hours the number of surviving L cells was determined by nuclear count. The cells were also studied in coverslip preparations stained with hematoxylin and eosin. The number of surviving L cells exposed to purified and crude suspensions of lymphoid cells from sensitized mice was significantly lower than the number surviving in the preparations with lymphoid cells from nonsensitized mice (Table I).

TABLE I. Effect of Lymphoid Cells on L Cells *in vitro* for 48 Hours.

Groups	No. of L cells* 1000/ml	± S.E.
L cells in medium 199 alone	86.7	2.8
Crude lymphoid cell suspensions		
L cells and control lymphoid cells	74.3	3.1
L cells and sensitized lymphoid cells	46.1	2.6
Purified lymphocyte suspensions (98% purity)		
L cells and control lymphocytes	75.5	3.9
L cells and sensitized lymphocytes	35.9	3.0

* Avg of 6 tubes in each group.

Comment. Various techniques have been employed for separation of leukocytes from mixed cell populations including sedimentation with or without addition of sedimenting agents, selective destruction of cells, and centrifugation. Most of the methods for isolation of cells from mixed cell populations have been devised for the isolation of cells from blood(3). Although several sedimentation methods were found to be satisfactory for separation of red cells and leukocytes from blood, this approach was unsatisfactory for removal of red cells from the crude suspensions of splenic tissue.

Because erythrocytes are more sensitive to hypotonic solutions than are lymphocytes, we used a hypotonic saline solution to remove the red blood cells. Mouse erythrocytes begin to undergo lysis at 0.535% NaCl and are completely hemolyzed at 0.33% NaCl(4). In the present study lysis of virtually all red cells occurred at 0.350% NaCl. Since the sensitivity of the erythrocytes may vary slightly with the species(4,5), sex(6) and age(4), changes in experimental conditions may necessitate a slight adjustment of the saline solution. The exposure to hypotonic saline and centrifugation did not demonstrably impair the viability or functional competence of the lymphocytes.

The purified suspension consisted predominantly of small and medium sized lymphocytes which accounted for 96% of the cells (range 90-99%). The remaining cells were macrophages and plasma cells; myeloid elements were rare in these suspensions.

The lymphocytes of the purified suspensions showed normal motility when studied by time-lapse microcinematography, and the

dye-exclusion test revealed insignificant numbers of dead cells. The brief exposure to unbuffered hypotonic NaCl solution did not impair the cytolytic activity of sensitized lymphocytes on homologous cells *in vitro*.

Summary. Highly purified suspensions of living, immunologically competent lymphocytes were obtained from splenic tissue of mice by destroying the red blood cells with hypotonic saline and separating the nucleated cells by centrifugation. The procedure yielded approximately 40 million lymphocytes from each spleen. Usually 96% (range 90-99%) of the cells were lymphocytes; the rest were other nucleated cells. The viability of lymphocytes was demonstrated by their motility and the exclusion of dye from their cytoplasm. The immunologic competence of these cells was shown by their cytolytic effect on homologous cells *in vitro*.

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A Comparison of Insulin Immunoassays.* (28836)

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All radio-immunoassays for plasma insulin are based on the use of the reaction between insulin-^{131}I and anti-insulin antibody as a tracer system for unlabeled insulin(1). However, different methods have been employed to separate the insulin-^{131}I-antibody complex from residual, "free" insulin. These include paper chromatography(2), salt precipitation (3), and immunoprecipitation(4). Furthermore, different periods of incubation have been utilized(2-4). Since widely divergent results have been obtained using these techniques(2-6), it is clear that some critical factors must exist which are not generally appreciated. In the present investigation some potentially critical factors have been studied: the methods of immunoprecipitation and chromatography have been compared with respect to the effect of interfering with the reaction equilibrium; the effect of dilution of the plasma, and the influence of the presence of *human* antibodies to insulin on the immunoprecipitation method have been assessed; and the immunological potency and stability of several human insulin reference standards over several years has been examined.

Materials. The guinea pig anti-beef antiserum (G.P.A.S.) was prepared by Dr. V. Jones using a Freund's adjuvant and Wellcome beef insulin. Rabbit anti-guinea pig γ-globulin serum (R.A.G.P.S.) was obtained from rabbits immunized to guinea pig γ-globulin emulsified in Freund's adjuvant. The guinea pig γ-globulin was precipitated from guinea pig normal serum at 37°C by addition of sodium sulphate to a final concentration of 13%. The precipitate was twice dissolved in 0.9% NaCl and reprecipitated with 12% sodium sulphate yielding 90% γ-globulin, 10% α_1-β globulin and no albumin on cellulose acetate electrophoresis. The same procedure was used to produce rabbit anti-human γ-globulin (R.A.H.S.). Beef insulin-^{131}I was prepared by adding 5 mc of io-

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