or no difference between the responses of mice receiving donor spleen cells incubated with saline alone or with the *E. coli*. However, when *E. coli*-incubated spleen cell suspensions were fractionated by centrifugation, there were marked differences in the number of BAC appearing in recipient spleens (Table). Many fewer BAC were present when the upper fractions, which were devoid of most bacteria adherent cells, were used for cell transfer. In contrast, the lowest fraction, which contained many more bacterial cluster forming cells, generally gave 2–4 times more BAC. This number of BAC, however, was generally similar to that which occurred following transfer of similar numbers of splenocytes which had not been incubated in vitro with the *E. coli*. The number of bacteriolytic PFC to *E. coli* in recipient spleens did not vary significantly, regardless of the treatment of the donor spleen cell suspensions or the type of fraction transferred (Table).

Incubation of normal spleen cells with sheep erythrocytes or *Shigella* resulted also in clusters of spleen cells and specific antigen, but there was no detectable effect on the number of BAC appearing in recipient spleens (Table). In addition, only very low numbers of BAC and PFC appeared in the spleens of X-irradiated control mice that were not given donor spleen cells. The appearance of antibody-forming cells in the recipients could be attributed to the donor cell population. Viable cells were necessary, since heating of the donor splenocytes at 80 °C for 1 h, regardless of in vitro treatment with antigen, resulted in a marked diminution in the number of antibody-forming cells, either BAC or PFC, that appeared in the recipients. Thus, it seems unlikely that any antigen transferred with the spleen cell suspension influenced the recipient response. Only those recipients actively challenged with the *E. coli* had significant BAC or PFC responses.

It seems reasonable from the results of these experiments to conclude that some of the splenocytes from normal mice which react with *E. coli* in vitro to form clusters may be directly involved in the immune response, either as precursors of antibody-forming cells or as antigen-reactive cells. The separation of such reactive cells with adherent bacteria from spleen cell suspensions by centrifugation markedly affected the ability of the remaining cell population to adoptively transfer antibody-forming capacity to X-irradiated syngeneic mice, as assessed by the BAC assay. However, there was little effect on the PFC response of the same recipients, which suggests that precursor or antigen-reactive cells for BAC are different from those for PFC, which depend on synthesis of lytic antibody. Specificity of the reaction suggests there are separate and distinct pre-existing cell populations that can respond to specific antigens. Thus the spleen cells from non-immunized mice that reacted in vitro with *E. coli* appeared to be the same ones that were concerned with the adoptive transfer of BAC formation, but not with development of PFC. However, these cells may merely represent immunocytes that persist after earlier stimulation by the same or cross-reacting antigens in the environment. The removal of these cells from a spleen cell suspension could thus decrease the number of 'memory' cells necessary for a secondary, rather than a primary type response to this bacterial antigen.

Zusammenfassung. Es gelingt durch niedertourige Differentialzentrifugierung normaler Milzzellen der Maus, in vitro mit *E.-coli*-Suspensionen inkubiert, den Grobteil der Vorläufer Antikörper bildender Zellen abzutrennen, welche zusammen mit *E. coli* spezifische Agglutinine bilden konnten. Dies war durch Erfassung bakterieller «Kolonieverklumpung» in Empfänger-Mäusen zu bestimmen.

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Passive Hemagglutination of *Herpesvirus hominis*

The efficacy of the passive hemagglutination (PHA) test in detecting antibody to viral antigens is well established[1]. It was reported in the literature[2,3] that *Herpesvirus hominis* can be passively coated on erythrocytes which will then be agglutinated by specific immune sera. A more recent publication[4] confirmed the effectiveness of the PHA test for detecting antibodies to *Herpesvirus* and also indicated that this method is effective in distinguishing between the 2 antigenic types of virus. The purpose of the work herein reported was to separate the antigen responsible for the PHA activity and to further characterize this test.

Materials and methods. Herpesvirus, type 1 MacIntyre strain VR 3, was grown in both HeLa and HEp-2 cells. The cells were grown as monolayers in either prescription bottles or in roller bottles and were harvested when the cytopathic changes were well advanced (3+). Infected cells were washed at least 3 times, resuspended in balanced salt solution (BSS) and stored at −70 °C. Before they were used all preparations were centrifuged for 10 min at $1500 \times g$ to remove cellular debris. Certain preparations were stabilized with formaldehyde[5]. Viral preparations were either purified on 5% to 50% sucrose gradients and centrifuged at 30,000 to 50,000 $\times g$ for 1 h or spun to equilibrium in CsCl self-forming gradients at $100{,}000 \times g$ for 44 h[6].

Fractions were routinely collected by the bottom drip method and diluted with approximately 4 volumes of phosphate-buffered saline, pH 7.2 (PBS). In certain

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experiments the band containing infectious virus was harvested by side puncture and tested undilute for antigenic activity.

Sera used to detect antigens were obtained from 3 sources: a) hyperimmune rabbit sera which were prepared by immunizing rabbits with virus grown in rabbit kidney cells[7], b) sera obtained from rabbits which developed encephalitis after immunization by scarifying the eye and dropping 0.1 ml of crude virus into the scarified area 6–10 days prior to bleeding and c) outdated human plasma obtained from our blood bank. Microtiter assays were used for detection of antigen and virus activity and included complement fixation (CF), passive hemagglutination, and infectivity procedures[1,7,8].

Results and discussion. Initial experiments were concerned with separation of the infectivity from the soluble antigens to determine whether the antigenic activity detected by passive hemagglutination was primarily associated with either the infectious virus or the soluble antigens. When the viral preparations were separated by sucrose rate zonal centrifugation, the antigenic activity was found exclusively on the top of the gradient with little separation of the PHA and CF activity. The infectivity was found considerably lower in the gradient and separate from the antigenic activity. Additional attempts were made to separate the CF from the PHA activity using longer centrifugations in sucrose density gradients. In these studies the infectious virus was removed by high speed centrifugation prior to separation on sucrose gradients and the supernatant was then centrifuged for 17 h at $100{,}000 \times g$. Little separation of the antigenic activity was accomplished.

Inability to separate the PHA from the CF activity on sucrose necessitated the use of equilibrium centrifugation in CsCl. After infectious virus was removed as described above, the complement-fixing activity was readily separable from the PHA activity, indicating that these tests apparently measure different antigenic moieties. The PHA reactive antigen was less buoyant than the antigen detectable by complement fixation methods. Some difficulty was occasionally encountered in localization of CF activity into a single peak, which probably indicates heterogeneity of CF antigens.

During these studies, interest was also directed to the host response to the soluble antigens. In the above data, various antisera reacted with the antigens and gave similar results. More definitive studies were initiated by reacting antisera with various antigen preparations in order to determine whether or not the test sera had identical specificities to the various antigen preparations tested. Approximately 30 human plasmas were collected and tested by both PHA and CF methods. It was noted that complete agreement between those 2 methods was obtained: all of the plasmas positive by 1 method were also positive by the other with good correlation between titers. The Table shows the results of a typical experiment. Included in the antigens tested were 2 PHA antigens and 1 CF antigen all of which were separated in CsCl gradients, an antigen pool remaining on the surface of a sucrose gradient and the opalescent band containing infectious virus. It can be seen that some variation in PHA activity was apparent between these sera; however, it was minimal. It also should be noted that in this experiment the concentrated virus band was positive in the CF test but negative in the PHA test. It was subsequently found that concentrated virus was reactive in the PHA test when used undilute or at low dilution (1:2). It was not determined if the PHA activity of the virus was a result of available reactive sites on the intact virion or viral degradation which uncovered active sites.

Antibody titers[a] to *Herpes virus hominis* soluble antigens

Antigen	Rabbit hyperimmune		Rabbit acute infection		Human	
	CF[b]	PHA[c]	CF	PHA	CF	PHA
G4F 6–7[d]	<10	<40	<10	640	<10	10,240
G5F 8–10[d]	20	640	<10	160	10	10,240
G5F 13–14[e]	40	<40	<10	<40	20	<40
SG5 B[f]	320	<40	160	<40	160	<40
SG8 T[g]	320	10,240	160	1280	80	20,480

[a] Expressed as reciprocal of dilution. [b] Complement fixation. [c] Passive hemagglutination. [d] PHA positive formalin treated antigens separated on CsCl gradients. [e] CF positive formalin treated antigens separated on CsCl gradient. [f] Virus 'band' separated by sucrose gradient. [g] Soluble antigens on top of sucrose gradient.

Zusammenfassung. Der passive Hämagglutinationstest (PHA) ist eine zuverlässige Methode für die Feststellung von Antigenen gegen *Herpesvirus hominis*. PHA-Testergebnisse mit denen des CF-Tests verglichen ergaben, dass die Antigene, die zur PHA-Aktivität führen, sich von denen der CF-Antigene und des infektiven Virus unterscheiden lassen.

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Daily Variations in Pigeon Cropsac Responses to Prolactin

Daily variations in responses to prolactin in several vertebrates have been described by our laboratory. For example, daily injections at one time of the day for 6 days resulted in increases (75–500%) in the lipid stores of fish[1], frogs[2], lizards[2], and birds[3], whereas injections at another time of day caused losses (20–60%) in the total body fat. Also, daily variations in responses to prolactin have been described for the growth of fish[1] and lizards[2], locomotor activity of birds[3], and the inhibition of amphibian metamorphosis[4]. Inasmuch as the pigeon cropsac response is the basis of prolactin bioassays, it seemed of interest to learn whether there is a daily variation in the sensitivity of the cropsac.

There are 2 fundamental techniques for assaying prolactin by the cropsac. The systemic (intramuscular) method[5] was tested during June, 1969, using 7–8-week-old white king pigeons. The intradermal method[6] was tested during December, 1969, using 4–5-month-old tumbler pigeons. For each test the birds of mixed sexes were caged in indoor metal coops; the temperature was maintained at 25 ± 2 °C, and a 12-h photoperiod (07.30 to 19.30 h) (100–200 lux at cage level) was supplied. The