Effects of Cortisol on Interferon Production by Mouse Peritoneal Leukocytes and Spleen *in vitro* and *in vivo*. (31413)

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Glucocorticoids have been shown to depress interferon production *in vitro*(1,2), *in ovo*(3-5) and *in vivo*(2), and reticuloendothelial cells may be an important site of interferon production during viral infection(6-10). The present studies were, therefore, undertaken to determine the effects of cortisol on interferon production by peritoneal leukocytes and spleen of mice.

Materials and methods. Cell lines. All cell cultures were propagated in Eagle's minimum essential medium (MEM)(11) containing 10 to 15% heat-inactivated fetal calf serum (FCS), chlortetracycline hydrochloride 50 μg (12), streptomycin sulfate 100 μg, and potassium penicillin G 100 units per ml. L cells (clone 929) were from an established line of mouse embryo fibroblasts(13). HeLa cells of the S-3 line were obtained from Dr. K. Habel, National Institutes of Health. Primary chick embryo fibroblasts (CEF) were prepared by trypsinization of decapitated 9- to 11-day-old embryos. Random bred mice were purchased from Carworth Farms, and CD-1 mice were purchased from Charles River Breeding Laboratories, Inc. Mouse peritoneal cells were obtained as follows: 20-30 g albino mice, male or female, were inoculated with 1.5 ml brain heart infusion broth (Difco) intraperitoneally and 24 hours later were sacrificed by cervical fracture. The abdomen was cleansed with iodine and alcohol, a skin flap was turned back to expose the peritoneal membrane; 10 ml of warmed MEM was injected into the peritoneal cavity and 7 ml of cell suspension removed by needle and syringe. Cell counts were made and Wright-stained smears were prepared for differential counts.

Viruses. Chikungunya virus (CV), a standard reference strain, was obtained from Dr. P. Russell, Walter Reed Arbovirus Unit. A stock of this virus was prepared from brains of infected suckling mice, harvested when symptoms appeared, and made into a 10% suspension (W/V) in MEM and assayed in CEF. Inactivation of CV was accomplished by incubation at 37°C for 24 to 48 hours and checked for infectivity in CEF. Encephalomyocarditis virus (EMC), a large plaque mutant (EMCr) originally obtained from Dr. K. K. Takemoto, N.I.H., was prepared and assayed in L cells. Vesicular stomatitis virus (VSV), Indiana strain, was obtained from American Type Culture Collection; stock supplies were prepared in CEF and assayed in L cells. Sindbis virus, originally isolated from a Malayan mosquito pool in 1956, was obtained from Dr. P. Russell and a stock of this virus prepared and assayed in CEF. A calf lymph strain of vaccinia virus, obtained from Dr. Karl Habel, was prepared and assayed in HeLa cells. Plaque assays were used to titrate all stock viruses except CV, which was titered in CEF and the $TCID_{50}$ calculated by the method of Reed and Muench(14). All stock viruses were maintained in a mechanical refrigerator at —80°C.

Interferon and control solutions were acidified with concentrated HCl to pH 2, kept for 24 to 72 hours, and then neutralized with 5 N NaOH. Interferon was assayed by the plaque reduction method(15) in L cells challenged with VSV(2). Titers of interferon were expressed as percent plaque reduction of control counts.

Hydrocortisone (cortisol) was employed as the sodium succinate (Solucortef, Upjohn).

Characterization of interferon. Interferon preparations were characterized as follows: 1) pH—stable at pH 2 up to 72 hours at 4°C; 2) temperature—partially inactivated at 56°C, and totally inactivated at 70°C for 30 minutes; 3) ultracentrifugation—no effect on antiviral activity after centrifugation at 100,000 × *g* for 2 hours; 4) proteolysis—

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complete loss of activity following treatment with 1:300 trypsin solution at 37°C for 30 min; 5) effect on extracellular virus—no inhibitory effect on VSV when solution and virus were mixed and incubated at 37°C for 30 minutes before plaquing; 6) species specificity—interferon solution did not reduce plaque counts of either VSV or Sindbis virus in CEF monolayers; 7) virus specificity—interferon induced by either EMC or CV reduced VSV plaque numbers in L cells; 8) washing of monolayer—no reduction in antiviral activity when L cell monolayers were washed 3 times with buffered saline after treatment with interferon solution prior to challenge with VSV.

Results. In vitro *effects of cortisol on interferon production by mouse peritoneal leukocytes.* Cell suspensions of mouse peritoneal leukocytes were collected in siliconized glassware (treated with Siliclad, Clay-Adams, Inc.). Plastic (Falcon) petri plates, 60 mm diameter, were each seeded with approximately 5×10^6 cells in 5 ml MEM containing 15% FCS. Cortisol was added to the appropriate cultures in concentrations varying from 1 to 100 μg/ml. The steroid was applied either at the same time as the virus inoculum, or 3 to 24 hours before, or 3 to 24 hours later. The leukocytes were inoculated with either CV or vaccinia virus; 4 to 24 hours later the cells were separated by centrifugation, and the supernatants prepared for interferon assay. A total of 22 experiments were conducted using a constant inoculum of virus in each experiment.

Data from a representative experiment with CV are summarized in Fig. 1. Cortisol neither increased nor decreased interferon production. Similar results were obtained with vaccinia virus. Neither the concentrations of cortisol nor the time when it was added affected these results. In each experiment control fluids from uninfected and untreated, as well as from uninfected and cortisol-treated cultures were included in the plaque reduction assays at the same dilutions as the interferon-containing fluids. Cortisol did not cause release of preformed interferon from uninfected cells.

EMC virus added *in vitro,* unlike CV and vaccinia virus, did not induce interferon production in peritoneal cell cultures although, as will be shown below, it was capable of inducing interferon in these cells when administered *in vivo.* Similar findings were noted by Glasgow(16). The reasons for this difference are not clear.

TABLE I. Total and Differential Cell Counts of Peritoneal Leukocytes.*

Group	Injections: Virus	Injections: Cortisol	Total cells/mm³	Differential counts,† %: P	L	Mo	Ma
I	+	0	1300	22	56	20	2
II	+	+	640‡	26	49	20	5
III	0	0	1240	15	61	19	5
IV	0	+	600§	28	52	16	4

* Cells harvested 26 hr after injection of 1.5 ml of broth i.p.

† P, polymorphonuclear leukocytes; L, lymphocytes; Mo, monocytes; Ma, macrophages.

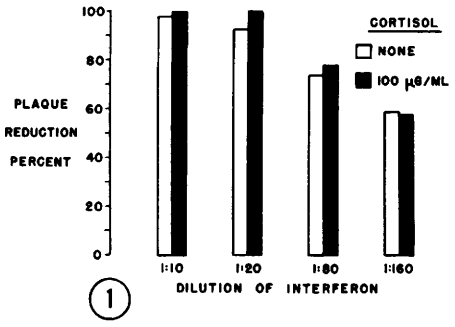
‡ Mean = 50.0 ± 4.3% of Group I ($P < 0.01$).

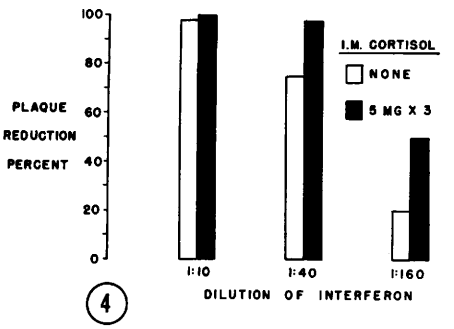
§ Mean = 50.6 ± 8.4% of Group III ($P < 0.01$).

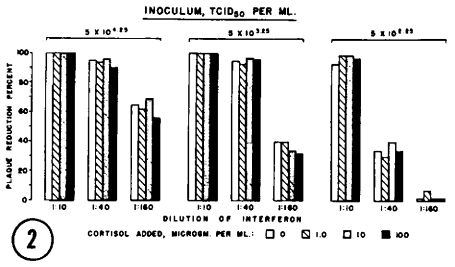
Differential and total counts of the peritoneal cells from these and other experiments are listed in Table I. Each differential cell count in the table represents the average of 5 separate counts of coded slides and additional checks by a second observer.

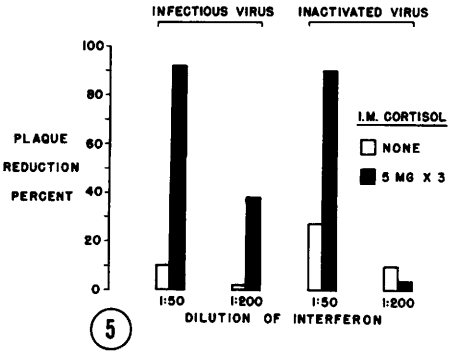
To determine whether the size of the inoculum of virus relative to that of cortisol would modify interferon synthesis by peritoneal cells *in vitro,* serial 10-fold dilutions of CV were were added to leukocyte cultures that had been exposed to cortisol 1, 10 or 100 μg/ml for 24 hours before challenge. The results of such an experiment are shown in Fig. 2. Interferon production by peritoneal leukocytes was not affected by cortisol over the 100-fold range of concentration of hormone and the 100-fold range of virus inoculum employed.

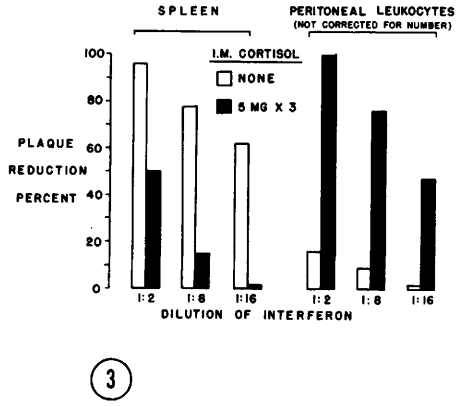
In vivo *effects of cortisol on interferon production by spleen and peritoneal leukocytes.* Mice were injected according to the schedule in Table II. Three animals were included in each group. Two hours after the final injections, that is, 26 hours after inoculation of EMC virus, mice in each group were sacrificed and peritoneal cells harvested. The cells in each group were pooled and divided among 3 petri dishes. Mean cell and differential counts are listed in Table I. Although cortisolized mice developed peritoneal exudates











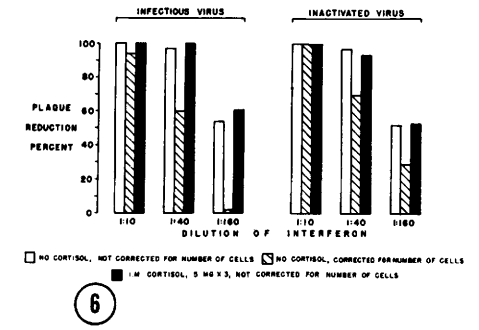


FIG. 1. Interferon production by mouse peritoneal leucocytes treated *in vitro* with cortisol 100 µg/ml 24 hr before inoculation with 5 x $10^{3.25}$ $TCID_{50}$ Chikungunya virus per ml. Samples obtained 24 hr after addition of virus.

FIG. 2. Effect of size of CV inoculum and of concentration of cortisol added *in vitro* on interferon production by mouse peritoneal leukocytes.

FIG. 3. Interferon production by mouse peritoneal leukocytes and spleen. Injections of cortisol and virus (EMC, 0.7 x 10^8 pfu per mouse) were administered according to the schedule shown in Table II. Explants were prepared 24 hr after injection of mice, and samples for interferon assays were obtained 24 hr after the cells were explanted.

FIG. 4. Interferon production by mouse peritoneal leukocytes. Saline, cortisol and broth administered to animals according to schedule in Table II. Infectious CV 5 x $10^{3.25}$ $TCID_{50}$ per ml added to explants 2 hr after final injections. Samples for interferon assays obtained 24 hr following addition of virus. Not corrected for number of cells.

FIG. 5. Interferon production by mouse peritoneal leukocytes. Saline, cortisol and broth administered to animals according to schedule in Table II. Results given for both infectious and inactivated CV (5 x $10^{3.25}$ $TCID_{50}$ per ml) added to leukocytes explanted 2 hr after the final injections, without correcting for number of cells.

FIG. 6. Interferon production by mouse peritoneal leukocytes. Saline, cortisol and broth ad-

ministered to animals according to schedule in Table II. Results, with and without correction for number of cells, are given for both infectious and inactivated CV ($5 \times 10^{5.25}$ $TCID_{50}$ per ml) added to explants 2 hr after injections.

TABLE II. Protocol for *in vivo* Effects of Cortisol on Interferon Production by Spleen and Peritoneal Leukocytes.

		Injections mouse		
Group*	Time, hr	Material	Amt	Route†
I	—24	Saline	.1 ml	i.m.
II	"	Cortisol	5 mg	"
III	"	Saline	.1 ml	"
IV	"	Cortisol	5 mg	"
I	0	Saline	.1 ml	i.m.
		Broth	1.5 ml	i.p.
		EMC	‡	s.c.
II	"	Cortisol	5 mg	i.m.
		Broth	1.5 ml	i.p.
		EMC	‡	s.c.
III	"	Saline	.1 ml	i.m.
		Broth	1.5 ml	i.p.
		Spent MEM	.5 ml	s.c.
IV	"	Cortisol	5 mg	i.m.
		Broth	1.5 ml	i.p.
		Spent MEM	.5 ml	s.c.
I-IV	+24	Same as —24 hr		

* 3 mice in each group.

† i.m., intramuscular; i.p., intraperitoneal; s.c., subcutaneous.

‡ 0.7×10^8 pfu/mouse.

containing approximately 50% fewer cells than saline-injected animals, corrections were not made in this experiment for numbers of cells per unit volume. Supernatant fluids from each culture plate were obtained at 2, 6 and 24 hours after the leukocyte explants had been prepared; the cells were removed by centrifugation and the fluids assayed for interferon. Control fluids in the interferon assays were from leukocyte cultures from uninfected mice injected with either cortisol or saline. There was no evidence of release of preformed interferon by cortisol from uninfected cells. Seven separate experiments were performed. Fig. 3 presents the data from one representative experiment; only results of the 24-hour harvest are shown here, but cortisol also markedly increased interferon production by the peritoneal cells when assayed after 2 and 6 hours as well.

In the same experiments production of interferon by splenic tissue was also determined. The average weight of the spleen from steroid-treated animals was approximately half that of noncortisolized controls. Spleens were aseptically removed, ground in a glass tissue crusher, and explants prepared in petri dishes containing MEM with 15% FCS. Supernatant fluids of the cultures were harvested after incubation at 37°C for 2, 6 and 24 hours. The results were similar for each of the 3 harvests. The findings are summarized in the left half of Fig. 3. No corrections were made for weight per unit volume in the explants. In contrast to the increased interferon production by peritoneal leukocytes, the interferon produced by splenic tissue of the cortisol-treated groups was significantly decreased over that from mice not treated with cortisol.

Gifford(17) has shown that optimal multiplicities of infection (MOI) exist for interferon synthesis. In the present study $5 \times 10^{4.25}$ $TCID_{50}$/ml. CV induced considerably more interferon in peritoneal cells than $5 \times 10^{2.25}$ $TCID_{50}$/ml (Fig 2). The augmenting effect of cortisol on interferon production by peritoneal cells may have been merely a consequence of the anti-inflammatory action of cortisol on the peritoneal membrane. Thus, cortisol may have promoted the optimal conditions for interferon synthesis by altering transfer of cells, virus or interferon, separately or in combination, across the peritoneal membrane. The following experiments were designed to explore some of these possibilities.

Effect of cortisol on viral replication in peritoneal cells and spleen. Mice were injected according to the schedule in Table II. Explants of spleen and peritoneal cells contained 7 ml of cell suspension per tissue culture plate. No corrections were made for cell numbers. At 30 minutes, 6 hours and 24 hours after the explants were prepared 5 ml of supernatant fluid was removed from one plate in each group for assay of interferon. The remaining 2 ml was used to wash the cells adhering to the plate into suspension. The suspension was frozen and thawed 4 times, and the cell debris removed by centrifugation (2,000 rpm for 10 minutes). EMC virus was assayed in L cells. The results (Table III) revealed no

TABLE III. Titers of EMC in Cultures of Peritoneal Leukocytes and Spleen Explants. (Animals injected according to schedule in Table II.)

Virus harvested, hr after explant*	Log_{10} titer of EMC in pfu/ml			
	Peritoneal leukocytes		Spleen	
	Saline	Cortisol	Saline	Cortisol
.5	2.30	2.57	2.81	3.30
6	2.18	2.61	2.00	3.15
24	<2.0	2.00	2.48	2.70

* From mice infected 26 hr previously.

significant differences between groups. Dickinson and Griffith(18) reported that EMC virus does not replicate in mouse peritoneal cells, and that was confirmed in this study.

Steroid administered in vivo, *virus infection* in vitro. Mice were injected according to the schedule in Table II except that instead of injecting the mice with EMC virus, cultures of peritoneal leukocytes were harvested 2 hours after the final injections and infected *in vitro* with CV, $5 \times 10^{3.25}$ $TCID_{50}$/ml. The results of such an experiment are presented in Fig. 4. No correction was made for numbers of leukocytes. Here again cells from animals receiving cortisol produced significantly more interferon than cells from other animals that did not. The differences were greater after correction for cell numbers.

That this effect was not a result of the action of cortisol on the virus, resulting in an optimal MOI for interferon formation, is indicated by two additional findings. First, the results noted in Fig. 4 are for interferon harvested 24 hours after adding virus, but similar results were obtained when interferon was harvested 4 to 6 hours after injection of cells; this is too brief a time to develop significant differences in titers of virus between cultures. Secondly, similar results were obtained when heat-inactivated CV was used as the inducing agent (Fig. 5).

The results, summarized in Fig. 4 and 5, reveal significant differences even without corrections for the number of leukocytes in the cultures. In Fig. 6 data are presented in which differences are observed only in the comparison between cultures corrected for the number of cells.

Discussion. It has been shown here that, in contrast to previous observations with fibroblast tissue(2), cortisol had no effect on interferon production when added to explanted peritoneal leukocytes. Furthermore, peritoneal cells harvested from the experimentally infected, cortisolized animals produced significantly greater amounts of interferon, although in the same animals less interferon was produced by splenic tissue. Moreover, peritoneal leukocytes from cortisolized mice produced more interferon than cells from non-cortisolized mice when the explants were infected with virus *in vitro*.

The role of interferon in recovery from viral infections has been extensively studied. Although it has been found to be important in certain infections *in vitro*, its role *in vivo* has not been established(19-21). Some attempts have been made to elucidate this role by experiments designed to reduce interferon levels in the host(22,23). Thus, the morbidity and mortality was increased in animals experimentally infected with virus and subjected to stress, and interferon levels in the serum of these mice were significantly depressed(22). Presumably the lower interferon levels in the stressed animals were a consequence of increased endogenous adrenal corticoid production, but this was not clearly demonstrated. In mice treated with 5 mg cortisol and infected 1 hour later with Sindbis virus intravenously the levels of interferon in the serum were significantly lower at 4 to 6 hours than in animals not pretreated with cortisol(2). These two experimental findings, namely: 1) that glucocorticoids depress early interferon production *in vivo*(2); and 2) that glucocorticoids in large doses increase susceptibility to, and virulence of, viral infections (24-28), suggest that the former may be the mechanism for the latter. However, direct experimental support for this hypothesis is still needed.

Evidence from numerous studies suggests that cells of the reticuloendothelial system (RES) and various leukocytes are important sites of production of interferon during viral infection(6-10). Since earlier studies have demonstrated that various steroid hormones were capable of depressing interferon production in fibroblast cultures and *in ovo*(2-5), it was considered possible that a similar effect

would be observed in cultures of peritoneal leukocytes. In the present studies cortisol, in concentrations ranging from 1 to 100μg/ml, when added to peritoneal cell explants up to 24 hours before challenge with vaccinia or CV failed to affect interferon production (Fig. 1). Moreover, in the experimental model employed, it was noted that peritoneal cells from cortisolized mice infected with EMC virus *in vivo* contained significantly greater amounts of interferon than cells from mice not treated with cortisol (Fig. 3). It was also observed that peritoneal leukocytes from cortisolized mice synthesized more interferon when explanted and infected with CV *in vitro*.

Why more interferon was formed by peritoneal cells when cortisol was given to the animals and not when the steroid was applied directly to the cells in culture is not clear. In both types of experiments, cortisol was introduced before, with and after the virus, but it is difficult or almost impossible to equate concentrations *in vitro* and *in vivo*.

The numbers of cells in the peritoneal exudate of cortisolized animals were usually about one half those in controls. The enhanced levels of interferon in the former were more striking when corrections were made for the numbers of cells in the cultures, and in some experiments enhanced production of interferon became evident only after such corrections (Fig. 6).

Administration of cortisol and EMC virus to mice enhanced synthesis of interferon by peritoneal leukocyte explants; however, splenic explants from the same animals produced less interferon than controls (Fig. 3). Although others have reported the decreased production of interferon in spleen after administration of glucocorticoids(29,30), peritoneal cells were not examined in those studies. Another recent study(31) revealed qualitative differences of interferon from peritoneal leukocytes as compared with interferon from the other sources (spleen and serum were tested), on the basis of sensitivity to 2-mercaptoethanol. Thus, the concept of "one species, one interferon" may require modification, for it now appears that there may be differences in the structure of interferons within a given species, depending on either the inducing agent(32) or the site of origin using the same inducer(31).

Smart and Kilbourne(33,34) recently expressed the view that "the regulation of infection may depend on a balance of the effects of endogenous corticosteroids on viral and interferon synthesis." They showed that hydrocortisone markedly augmented interferon synthesis after 96 hours in the chick embryo infected with Lee influenza virus that had been partially inactivated by exposure to ultraviolet light. They postulated that hydrocortisone inhibited early interferon production, leading to an increase in viral titer which, in turn, resulted in enhanced interferon formation.

In the present studies these and other possibilities were considered in an attempt to explain the phenomena observed with the peritoneal leukocytes: 1) Glucocorticoids affect the transfer of cells, and perhaps of virus, across the peritoneal membranes by modifying the inflammatory response(35). Thus, the observed phenomenon may have been a consequence of the optimal MOI for interferon production existing in the peritoneal cavity of the steroid-treated animals. However, the enhanced interferon production noted when peritoneal cells from steroid-treated animals were challenged *in vitro* with CV, would suggest that this was not the underlying mechanism. 2) The phenomenon may have reflected the effect of the steroid on viral replication resulting in the optimal MOI for interferon synthesis. Since the effect was noted as early as 4 hours after the inoculation of either infectious or inactivated CV, this would appear unlikely. 3) Cortisol, unlike endotoxin(23), did not cause release of preformed interferon from uninfected cells. Therefore, the differences cannot be explained on this basis. 4) The types of cells obtained from the different groups of animals did not differ greatly. 5) Hydrocortisone may modify peritoneal leukocytes to synthesize greater amounts of interferon. Further attempts to elucidate the behavior of peritoneal cells are currently in progress. Steroids other than glucocorticoids are being tested to see if they augment interferon production. Methods for separating the various cells in the mixed

population of peritoneal leukocytes are being employed in an attempt to observe the behavior of the individual cell types.

Interferon may be an important determinant of recovery from viral infection. If cells of the RES play a significant role in the elimination of virus from an infected host, then the effect of cortisol on interferon production by reticuloendothelial cells may be important in the pathogenesis and course of viral infection.

Summary. Interferon production by mouse peritoneal leukocytes infected *in vitro* was unaffected by addition of cortisol *in vitro*. Peritoneal cell explants from infected mice treated with cortisol produced significantly greater amounts of interferon, although less interferon was produced by splenic tissue of the same mice. Enhanced interferon production by peritoneal cell explants from cortisol-injected animals was also observed when the cells were infected with virus *in vitro*. The possible significance of these data is discussed.

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1. DeMaeyer, E., DeMaeyer, J., Nature, 1963, v197, 724.
2. Mendelson, J., Glasgow, L. A., J. Immunol., 1966, v96, 345.
3. Kilbourne, E. D., Smart, M. K., Pokorny, B. A., Nature, 1961, v190, 650.
4. Reinicke, V., Acta Path. Microbiol. Scand., 1964, v60, 528.
5. ———, ibid., 1965, v64, 339.
6. Gresser, I., Proc. Soc. Exp. Biol. and Med., 1961, v108, 799.
7. Glasgow, L. A., Habel, K., J. Exp. Med., 1963, v117, 149.
8. Ho, M., Bact. Rev., 1964, v28, 367.
9. Kono, Y., Ho, M., Virology, 1965, v25, 162.
10. Glasgow, L. A., J. Exp. Med., 1965, v121, 1001.
11. Eagle, H., Science, 1955, v122, 501.
12. Hayflick, L., Chanock, R. M., Bact. Rev., 1965, v29, 185.
13. Sanford, K. K., Earle, W. R., Likely, G. D., J. Nat. Cancer Inst., 1948, v9, 229.
14. Reed, L. J., Muench, H., Am. J. Hyg., 1938, v29, 493.
15. Wagner, R. R., Bact. Rev., 1960, v24, 151.
16. Glasgow, L. A., personal communication.
17. Gifford, G. E., Nature, 1963, v200, 91.
18. Dickinson, L., Griffith, A. J., Brit. J. Exp. Path., 1966, v47, 35.
19. Glasgow, L. A., Habel, K., J. Exp. Med., 1962, v115, 503.
20. Baron, S., Advances in Virus Research, Academic Press, New York, 1963, p39.
21. Glasgow, L. A., J. Pediat., 1965, v67, 104.
22. Chang, S., Rasmussen, A. F., Nature, 1965, v205, 623.
23. Ke, Y. H., Singer, S. H., Postic, B., Ho, M., Proc. Soc. Exp. Biol and Med., 1966, v121, 181.
24. Kass, E. H., Finland, M., Ann. Rev. Microbiol., 1953, v7, 361.
25. Kass, E. H., Lundgren, M. M., Finland, M., J. Exp. Med., 1954, v99, 89.
26. Kass, E. H., Finland, M., Adv. Int. Med., 1958, v9, 45.
27. Cheatham, W. J., Weller, T. H., Dolan, T. F., Dower, J. C., Am. J. Path., 1956, v32, 1051.
28. Haggerty, R. J., Eley, R. C., Pediatrics, 1956, v18, 160.
29. Rytel, M., Kilbourne, E. D., J. Exp. Med., 1966, v123, 767.
30. Postic, B., personal communication. (I.E.G. #6, Interferon Memo 146).
31. Lackovic, V., Borecky, L., personal communication. (I.E.G. #6, Interferon Memo 171).
32. Youngner, J., Stinebring, W., Hallum, J., personal communication. (I.E.G. #6, Interferon Memo 150).
33. Smart, K. M., Kilbourne, E. D., J. Exp. Med., 1966, v123, 299.
34. ———, ibid., 1966, v123, 309.
35. Greenberg, A. D., Morgan, H. R., N. Y. State J. Med., 1961, v61, 455.

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