gated. The results were compared with previously reported studies in which autologous blood was infused.

The size of the total blood granulocyte pool and the distribution of cells in the circulating and marginal compartments were normal in all 12 of the subjects studied. However, in 5 of the 12 subjects the half-disappearance time was more rapid than normal. It is suggested that in these 5 subjects, naturally occurring granulocyte incompatibilities may have accounted for the rapid disappearance of cells from the circulation.

Labeled isologous granulocytes migrated into inflammatory exudates less readily than did labeled autologous granulocytes. This observation may be of significance in regard to the therapeutic efficacy of isologous leukocyte transfusion.

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Erythropoietic Stimulating Factor (ESF) Associated with an Oncogenic Virus-Host System *in vitro*.* (30601)

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The existence of an erythropoietic stimulating factor or factors (ESF) or erythropoietin has been established beyond reasonable doubt(1,2,3). Recently ESF has been reported to stimulate cell division or growth both *in vivo*(4) and *in vitro*(5). The former report was based on experiments with Novikoff hepatomas in rats. The latter report concerned cultures of bone marrow cells from a human monocytic leukemia patient and human synovial membrane cells.

Several workers have reported the association of increased concentrations of ESF or situations compatible with an increased level of ESF with different types of cancer. Remission of associated polycythemia has been reported to occur following surgical removal of 2 malignancies(6,7). An ESF has been reported in fluid from a cystic cerebellar hemangioblastoma(8). Tumor growth was also reported to be related to the process of

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erythropoiesis by Eilers de Cousser(9).

Tobiska and Brada(10) noted increased ESF concentration in plasma of rats during some "stages" of growth of the Jensen tumor. Leaders(11) reported a similar increase in ESF activity in extracts of pooled plasma from humans with various forms of cancer. The possibility of a relationship between malignancies and blood levels of ESF therefore is evident.

Many forms of cancer in many species of animals are known to be caused by viruses (12). Induction of malignant tumors in hamsters by injection with human adenovirus is well established(13,14,15). The possibility that an ESF-like compound might be involved in the relationship between tumor and virus is tested in the present report.

Materials and methods. Four primary experimental situations were studied. They are as follows: (A) Adenovirus, type 12 in hamster cell culture, (B) Adenovirus, type 4 in hamster cell culture, (C) Adenovirus, type 12 in monkey cell culture, (D) Adenovirus, type 4 in monkey cell culture.

Viruses. Human adenovirus types 4 and 12 were used. Both types were obtained through the courtesy of Dr. McCullough of the Communicable Disease Center Field Station at Kansas City, Kansas. The Adenovirus 12 used in this study was from the American Type Culture Collection ET 981. It was grown in secondary monkey kidney cells at the time of the present experiment. Adenovirus 4 was isolated from a patient at this hospital and cultivated in Wistar Line 38 cells (human fetal lung, fibroblastic) obtained from Flow Laboratories, Rockville, Md.

Cells and media. Cultures of 2 cell lines were used. Hamster kidney cells were cultured in this laboratory from weanling hamsters using the method and medium described by Kissling and Reese(16). Monkey kidney cells were obtained from Dr. Herbert A. Wenner, Dept. of Pediatrics, University of Kansas Medical Center. High cystine altered Eagle's medium(17) was used to support these cultures.

Titration of adenovirus types 4 and 12. HeLa cells grown in Eagle's minimum essential medium (MEM) with 10% newborn calf serum were used to titer the viruses before and after they were grown for 4 days in primary hamster and monkey kidney cells. Serial 10-fold dilutions of virus were prepared in Eagle's MEM with 3% calf serum and 4 roller tube monolayer cultures of HeLa cells were inoculated with 0.1 ml of each dilution. Following incubation at 37°C for 5 days, titers were determined using standard titration methods(18). Virus titers which were inoculated into cultures of both monkey and hamster kidney cells were as follows: Adenovirus, type 4—10^8 $TCID_{50}$/0.1 ml. Adenovirus, type 12—10^7 $TCID_{50}$/0.1 ml.

Culture inoculation and preparation. Cells were grown in Roux bottles containing 100 ml of medium. On the seventh day of growth the growth medium was replaced with maintenance medium and 10 ml of virus was inoculated into the cultures. Four Roux bottles of each cell type were inoculated with each virus. On the fifth day after inoculation, cells were scraped into the medium and cells and medium were harvested. Only minimal cell degeneration was observed in any of the cultures. This is consistent with the report of Paul in 1960(19). Non-inoculated cells and medium were harvested in the same manner to serve as cell controls. Fresh medium and medium which had been incubated at 37°C for 5 days with an inoculum of virus equal to that added to the cell cultures served as medium and medium plus virus controls. The cultures and/or media were frozen until time of extraction.

Preparation of extracts of cell culture media. The extracts were prepared by a modification of the method described by Borsook *et al*(20) which has been described previously in detail(4). Rats received subcutaneously (s.c.) 2 ml (high dose) or 1 ml (low dose) of these extracts daily for 3 successive days prior to Fe^{59} injection.

In other studies, samples of active extracts were inactivated by one of 2 methods. One group was stored at room temperature for approximately 30 days. This has been reported to inactivate ESF(5). Another group was denatured by acid and heat by the method of Rambach *et al*(21).

Assay for ESF activity. ESF activity was

measured using red cell Fe^{59} uptake. This method, the fasted rat assay, has been reported previously(22) and has been used with modification as follows. Siminson rats weighing between 180 and 250 g were used to obtain the values reported. Food was removed from the cages at the beginning of the experiment, water was furnished *ad libitum*. Extracts were injected subcutaneously in equally divided doses on days one, two and three. Fe^{59} (1 μC) was injected intraperitoneally as ferrous citrate at the time of administration of the last dose of extract on day 3.

Animals were weighed and etherized 24 and 48 hours after injection of Fe^{59} (days 4 and 5). Samples of blood (0.6 ml) were obtained by cardiac puncture. Aliquots of 0.1 ml were used in determination of duplicate microhematocrits which were averaged to obtain the values used. Samples containing 0.5 ml of blood or 1.0 ml Fe^{59} citrate standards were counted using a Packard Gamma well type scintillation detector and recorded *via* a Packard Tricarb Spectrometer.

The Fe^{59} incorporation in animals following administration of the various extracts was compared with that in control animals which were not injected with extract. ESF extracts from the Armour Pharmaceutical Co., obtained through an allocation of the Erythropoietin Committee of the National Heart Institute, were also included in each run to insure that the test animals were responsive to exogenous ESF.

Per cent Fe^{59} uptake was calculated using a modification of the method described by Hennessey and Huff(23). The formula used for these calculations is

$$\frac{\text{A.W.}}{100} \times 6.80 \times \text{HCT} \times \frac{\text{CPM/ml}}{\text{T.C. Inj.}} \times 100 = \% \text{ uptake.}$$

A.W. = animal weight in g; 6.80 = ml blood per 100 g rat body wt(24); HCT = hematocrit; CPM/ml = counts per minute per ml of rat blood (less background); T.C. Inj. = total counts per minute injected (less background).

Data were prepared for submission to the computation center at the University of Kansas. Per cent Fe^{59} uptake values, group mean values and confidence limits were determined *via* IBM 7040 computer. The "student t" test, also done by computer, was used throughout this work to evaluate difference between values. A p value of less than 0.05 was regarded as significant.

Results. The effect of injection of extracts of the media from the 4 experimental combinations of adenovirus and cell culture of Fe^{59} uptake is illustrated in Fig. 1. Significant ESF activity was found at both dose levels only in extracts of hamster cell cultures inoculated with adenovirus, type 12. Much less ESF activity was present in extracts of hamster cell culture inoculated with adenovirus 4. Fe^{59} uptake following the large dose (2 ml) of this extract was significantly greater than for control animals. An inhibitory effect on Fe^{59} uptake was demonstrated for the extracts of cultures of monkey cells inoculated with either type of adenovirus.

Fig. 2 depicts the effects of extracts of the various components of the hamster cell, adenovirus 12 culture on Fe^{59} uptake. Again, ESF activity was found in extracts of hamster cell cultures inoculated with adenovirus 12. Significant ESF activity was present when large (2 ml) doses of medium and/or virus and cells were injected. When 1 ml injections (small dose) were injected, however, only the combination of virus, cells and medium produced Fe^{59} incorporation significantly greater than that recorded for control animals.

Fig. 3 illustrates the effects of extracts of the various components of the monkey cell, adenovirus 4 cultures on Fe^{59} uptake. ESF activity could be demonstrated only in extracts of medium plus cells. Fe^{59} uptake was significantly less in animals treated with extracts containing adenovirus 4 in medium or adenovirus 4 and monkey cells in medium than that recorded for the control group. The magnitude of the inhibition was not as great, however, as that recorded in the experiments depicted in Fig. 1.

Following injection of extracts which had been inactivated by either storage or heat, values for Fe^{59} uptake were not significantly different from control animals. Neither the

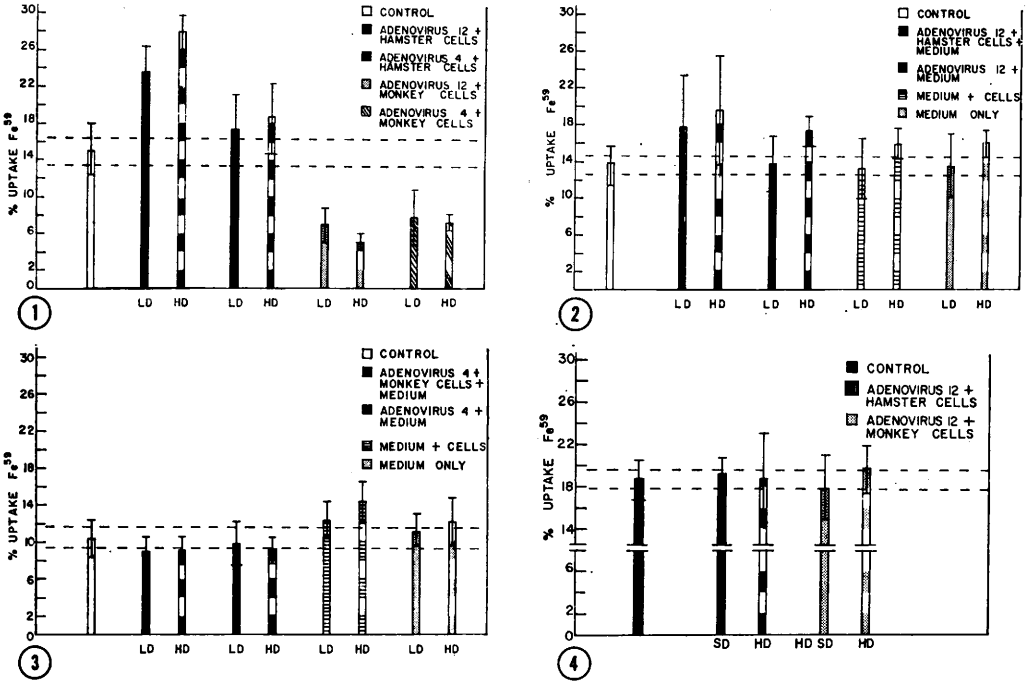

FIG. 1. Effects of administration of extracts of cell culture media on Fe^{59} uptake by red blood cells in rats. L.D., 1 ml of extract daily; H.D., 2 ml of extract daily. Broken horizontal lines are S.E. of control values. Values are mean values with 95% confidence intervals.

FIG. 2. Effects of administration of extracts of various components of cultures of hamster cells on Fe^{59} uptake by red blood cells in rats. L.D., 1 ml of extract daily; H.D., 2 ml of extract daily. Broken horizontal lines are S.E. of control values. Values are mean values with 95% confidence intervals.

FIG. 3. Effects of administration of extracts of various components of cultures of monkey cells on Fe^{59} uptake by red blood cells in rats. L.D., 1 ml of extract daily; H.D., 2 ml of extract daily. Broken horizontal lines are S.E. of control values. Values are mean values with 95% confidence intervals.

FIG. 4. Effect of denaturing on the action of extracts of cell culture media on Fe^{59} uptake by red blood cells in rats. S.D., inactivated by storage at room temperature for 30 days; H.D., denatured by acid and heat. Broken horizontal lines are S.E. of control values. Values are mean values with 95% confidence intervals.

stimulatory nor the inhibitory effects recorded previously were noted. This is illustrated in Fig. 4.

Discussion. Human adenovirus, type 12, is known to be oncogenic when injected into newborn hamsters(13,14,15). Human adenovirus, type 4, however, has been reported not to be tumorigenic when injected into hamsters(25). Neither type of adenovirus has been reported to be tumorigenic in monkeys. Consequently, type 12 adenovirus grown in cultured hamster cells was chosen as a system which could produce an ESF-like substance if one were associated with the process of tumor induction. Adenovirus type 4 growing in cultured hamster cells and both types of adenovirus growing in cultured monkey cells were chosen as culture systems in which ESF would probably not be found if this substance were associated specifically with tumorigenesis.

Extracts of cultures of hamster cells inoculated with human adenovirus, type 12, have been shown to contain an ESF as measured by the Fe^{59} uptake method. The occurrence of large amounts of ESF only in the experimental situation *in vitro* analogous to that *in vivo* which results in tumor formation is suggestive that ESF may be associated with cell division in both situations. The finding that extracts of hamster cell cultures inoculated with adenovirus 4 produced

a weak ESF effect suggests that adenovirus 4 may ultimately prove to be weakly oncogenic in hamsters.

The depression of Fe^{59} uptake by extracts of monkey cell cultures was not significant when Fe^{59} uptake of control animals was low. Thus there is apparently a minimal Fe^{59} uptake activity which may occur. This "inhibition" of Fe^{59} uptake suggests at least two possibilities. First, a substance which depresses bone marrow activity may be produced in these cultures. Second, some material in the medium itself or the monkey cells may inhibit Fe^{59} uptake. The first possibility appears more likely in light of "inactivation" experiments discussed below.

Since the nutritional state of the animals is known to influence the Fe^{59} uptake in the "starved rat" assay, the possibility that the increase in Fe^{59} uptake was the result of the nutritional material in both media was tested. When the components of the hamster cell cultures were tested, extracts of medium alone, medium and hamster cells and medium and virus did result in significant increases in Fe^{59} uptake when tested in high doses (2 ml). Extracts of medium from cultures containing both hamster cells and adenovirus 12, however, resulted in significant increases in Fe^{59} uptake following both low and high doses. This suggested that an ESF effect greater than that due to nutritional factors was obtained.

Testing of the components of monkey cell cultures did not result in increased Fe^{59} uptake. Administration of extracts of adenovirus and medium or adenovirus, monkey cells and medium resulted in a smaller uptake of Fe^{59} than was observed in control animals.

To provide additional information as to mechanism of these ESF effects some extracts were subjected to procedures known to inactivate ESF. Sulfuric acid plus heat(21) and storage at room temperature(5) were used. Both of these procedures abolished the stimulatory activity of extracts of cultures of adenovirus 12 with hamster cells as well as the inhibitory activity of the monkey cell culture extracts. This suggests specific hormone-like stimulatory and inhibitory factors in the extracts from these cultures.

The presence of an erythropoietic stimulating factor (ESF) in the extracts from these cell cultures is consistent with the hypothesis, suggested previously, that ESF is actually a nonspecific growth factor (NGF). Further, the concomitant occurrence of an inhibitory factor in the media from monkey cell cultures suggests a similarity to the substances "promine" (growth promoting) and "retine" (growth inhibiting) which have been reported by Hegyeli *et al*(26) and Szent-Gyorgyi *et al* (27). Based on *in vitro* experiments(5) these substances have been postulated to be members of a closely related family of nonspecific growth controlling substances.

The association of a nonspecific growth factor (NGF, ESF) with a combination of virus and cell system *in vitro* which is known to be oncogenic *in vivo* suggests a possible role for this substance in tumorigenesis. The possibility that a cause-effect relationship exists between the production of NGF (ESF) by a virus-host cell system and the ability of this system to produce a malignancy *in vivo* should be investigated further.

Summary. The possibility that erythropoietic stimulating factor (ESF) might be involved in the relationship between tumor and virus was tested *in vitro*. Four experimental situations were studied: (A) Adenovirus, type 12 inoculated into hamster cell culture, (B) Adenovirus, type 4 into hamster cell culture, (C) Adenovirus, type 12 into monkey cell culture and (D) Adenovirus, type 4 into monkey cell culture. Each of these cultures was extracted and assayed for ESF. In addition, other cultures containing only virus or cells in medium or medium alone were extracted and assayed.

Extracts of cultures of hamster cells inoculated with human adenovirus, type 12, were found to contain an ESF. This material was found only in small amounts in the other experimental situations. The occurrence of this ESF primarily in the experimental situation *in vitro* analogous to that *in vivo* which results in tumor formation suggests that an ESF-like material may be associated with the process of cell division in both situations.

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Influence of Actinomycin D, Aurantine and 5-Bromdeoxyuridine on Production of Nucleic Acid and Protein in Measles Virus. (30602)

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Previous experiments have established that viral RNA itself is the matrix for viral RNA synthesis and not the cellular DNA. Substantiating these conclusions are numerous experiments with actinomycin D, an antibiotic which forms a complex with the DNA molecule(1) thereby inhibiting the activity of RNA-polymerase and of the cell RNA synthesis; to a lesser extent this is also true for the DNA-polymerase and DNA synthesis (2,3). Experiments with actinomycin D have shown that the reproduction of RNA-containing viruses—Mengo(4), Newcastle Disease (5), phage MSE(6), encephalomyocarditis of mice(7), Sendai(8) and Venezuelan equine encephalitis(9)—is not dependent on the cell DNA whereas the protein and nucleic acid synthesis of RNA-containing influenza virus depended upon the cell DNA(5,8).

That DNA does not participate in the synthesis of the majority of RNA-containing viruses is also confirmed by the fact that 5-bromdeoxyuridine and other thymine analogs which inhibit the synthesis of the cellular DNA and viral DNA do not inhibit reproduction of the RNA-containing viruses, such as poliomyelitis(10,11), vesicular stomatitis (10), arboviruses(11,12,13), Newcastle Disease(13) and others. Inhibition by 5-fluordeoxyuridine and by actinomycin D of the RNA-containing Rous sarcoma virus is explained by the influence of the viral RNA