EFFECTS OF SERUM ON MEMBRANE TRANSPORT

I. Separation and Preliminary Characterization of Factors Which Depress Lysine or Stimulate Adenosine Transport in Rabbit Alveolar Macrophages*

By PHYLLIS R. STRAUSS‡ and RICHARD D. BERLIN§

(From the Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115)

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Serum has long been known to affect the longevity and reproductivity of mammalian cells in tissue culture (1–5). These observations are thought to reflect the in vivo milieu where all cells live in a protein-rich environment providing nutrients and regulators (6). Recently several studies have shown that serum affects the permeability of cells to amino acids and nucleosides and it was suggested that these alterations were important in regulating cell growth and reproduction (7–11).

In this report we have examined the effects of serum on a nucleoside and an amino acid membrane transport system in rabbit alveolar macrophages. Each has already been extensively characterized by Tsan and Berlin (12, 13) by means of a rapid sampling technique (14). This technique has enabled us to examine the effects of serum on initial velocities of uptake and thus on membrane transport as opposed to intracellular metabolic events. Short preincubations with normal rabbit serum result in specific but opposite effects on the two systems. The serum components responsible have been partially characterized and shown to be different.

Materials and Methods

Rabbit lung macrophages were obtained as previously described (12). Sera or plasma from normal white rabbits of either sex were obtained by heart puncture and stored frozen in small samples at −20°C. They were tested for ability to affect lysine and adenosine transport by the cover slip technique developed by Hawkins and Berlin (14). Briefly, monolayers were formed from freshly obtained lung macrophages on circular 22 mm cover slips. Each monolayer contained 0.3×10^6 cells. The monolayers were exposed to the material to be tested for 30 min, unless otherwise specified. They were then rinsed through four changes of modified Hanks' solution (MH)[1] (14) at room temperature and then tested for ability to transport either lysine

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[1] *Abbreviations used in this paper:* AEA, adenosine-enhancing activity; gamma G, gamma globulin; LDA, lysine-depressing activity; MH, modified Hanks' solution; MIF, migration inhibitory factor; NRS, normal rabbit serum.

(0.1 mM, 1 μCi/ml) or adenosine (0.04 mM, 2 μCi/ml) during a 45 s incubation period. Every determination was run in quadruplicate. The concentrations of permeant were chosen at their *Km* values (12, 13). Isotopes were obtained from New England Nuclear, Boston, Mass., or Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. No serum was present during the transport assay except substances remaining adherent to the macrophage monolayers after thorough washing. The amount of freshly chromatographed [^{14}C]inulin included during the serum preincubation or during preincubation with buffer alone that remained on the monolayer after washing was insignificant, corresponding to less than 1% of the amount of the isotope transported by the monolayers.

RESULTS

Dose and Time Dependency of Serum Effects.—Preincubation of macrophage monolayers with normal rabbit serum (NRS) significantly ($P < 0.01$) depressed lysine from control values. Maximal depression of different sera usually occurred at 0.5% and 1.0% NRS, at which doses treated monolayers transported lysine at a rate of 59 ± 2.6% (SE, 31 observations) of the control rate. Considerable variability in depression was obtained at higher concentrations of serum. Fig. 1 (top) represents a typical dose-response curve. All rabbit sera and plasma had the activity. All cell preparations were sensitive. The lysine-depressing activity will be referred to as LDA.

Preincubation with NRS diluted to 5% significantly ($P < 0.01$) elevated

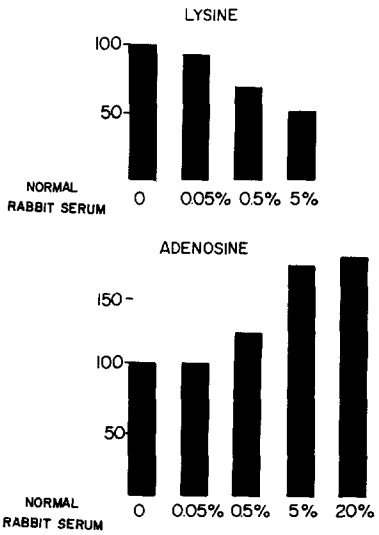

FIG. 1. Concentration dependence of serum effects. (*Top*) Lysine. Preincubation for 30 min with 0.5% NRS depresses lysine in this experiment to 60% of controls preincubated with MH. Values are expressed as percent of controls preincubated with MH. Control cover slips transported 0.386 nmol lysine/10^6 cells per min. Preincubation with 5% serum further depresses lysine transport. However the depression induced by 5% or higher concentrations of NRS was variable from experiment to experiment. It was often as little as 10–15%. (*Bottom*) Adenosine. Preincubation for 30 min with 5–20% NRS stimulates adenosine transport as much as 75% of controls preincubated with MH. Values expressed as percent of controls preincubated with MH. Controls transported 0.273 nmol adenosine/10^6 cells per min.

adenosine transport by 35 ± 2.4% (SE, 60 observations) of the control values. A typical dose-response curve is shown in Fig. 1 (bottom). Sera from 14 of 19 rabbits had the activity. The failure of some sera to produce stimulation did not seem to be related to sex or to the time of day when blood was drawn. In addition, a serum with good activity occasionally produced no, or marginal, stimulation of adenosine transport. Three individual sera repeatedly assayed resulted in the following enhancement of adenosine transport: (*a*) 34 ± 4.5% (SE, 6 experiments), (*b*) 34 ± 4% (SE, 18 experiments), (*c*) 59 ± 6% (SE, 6 experiments). The values obtained with the second serum ranged from 5 to 59%; the values obtained with the third serum ranged from 40 to 81%.

There are various materials present on the surface of the lung, including surfactant (15), which might alter responsiveness. However, washing cells several times after they were obtained from the lung did not alter the response. In other experiments addition of the fluid obtained from lung lavage to the active serum did not alter the degree of adenosine stimulation, nor did the lavage fluid itself affect adenosine transport as compared with controls preincubated with MH. When freshly obtained sera were initially assayed for activity, a serum with known activity was always included in the experiment. If the known serum produced a good response, new sera tested in such an experiment which showed no activity were discarded. The adenosine-enhancing activity will be referred to as AEA.

The times of preincubation with serum required for LDA and AEA are shown in Fig. 2. Maximal LDA was obtained after 30 min and maximal AEA

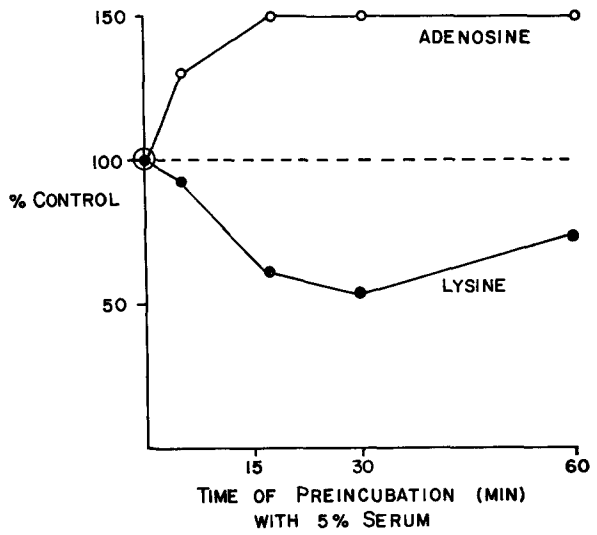

FIG. 2. Effect of duration of NRS preincubation on LDA and AEA. Preincubation for 30 min maximally depressed lysine transport from control values. Preincubation for 15 min maximally enhanced adenosine transport. In each case the effects are given as the percent of control cover slips preincubated with MH for the same lengths of time.

was obtained after preincubation for 15 min. If serum was removed from the monolayers by washing them thoroughly and they were further incubated with MH, both LDA and AEA were retained for more than 40 min.

Kinetic Effects.—In order to define the effects of serum on kinetic parameters of each transport system, monolayers were pretreated with MH or NRS and concentration vs. velocity experiments were performed (Figs. 3 and 4). In each

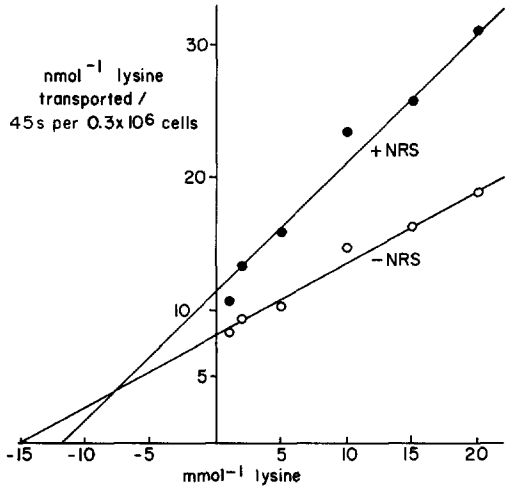

FIG. 3. Reciprocal plot of lysine transport with (●) and without (○) preincubation for 30 min with 0.5% NRS. Lines drawn by least squares method. An alteration in V_{max} from 500 pmol/min per 10^6 cells to 352 pmol/min 10^6 cells can be calculated. The small change in *Km* could not be confirmed in other experiments or by plotting these data as suggested by Dowd and Riggs (16).

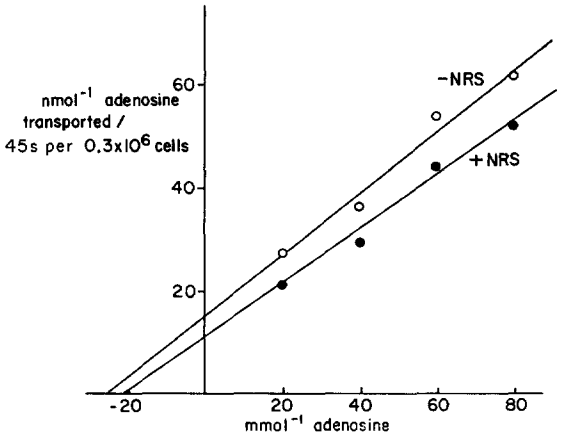

FIG. 4. Reciprocal plot of adenosine transport with (●) and without (○) preincubation for 30 min with 5% NRS. Lines drawn by least squares method. An alteration in V_{max} from 268 pmol/min per 10^6 cells to 364 pmol/min per 10^6 cells was effected. As in the case of serum-induced alterations in lysine transport, the change in *Km* could not be confirmed.

case serum altered the maximal velocity. Effects on the affinity constant could not be definitively demonstrated, even when the data were plotted in the different ways suggested by Dowd and Riggs (16).

Exchange Diffusion.—An immediate concern regarding the lysine transport system is the effect of serum on internal pools of free lysine. An increase in pool size may result in increased transport rate, a phenomenon commonly called exchange diffusion (17, 18). Therefore, experiments were performed to determine whether alterations in internal lysine concentrations affected LDA. Monolayers were preincubated with 0.5% NRS or with MH. After being rinsed thoroughly, they were postincubated with 10 mM lysine, 0.3 μCi [^{14}C]lysine/ml, or with MH. The results of two experiments are given in Table I. Whether or

TABLE I

Effect of Serum on Lysine Transport Measured after Loading with 10 mM Lysine

Incubations		Accumulation		Transport	
Precincubation	Postincubation	nmol lysine/μl cell water per 15 min	% MH control	pmol lysine/μl cell water per 45 sec	% MH control
NRS	MH			56.9	76
MH	MH			74.0	
NRS	10 mM lysine	9.2	92	170.5	73
MH	10 mM lysine	10.0		230.8	
NRS	MH			35.3	67
MH	MH			52.6	
NRS	10 mM lysine	5.2	100	75.3	74
MH	10 mM lysine	5.3		101.1	

Two typical experiments are shown. Monolayers were preincubated with 0.5% NRS or with MH. After being rinsed thoroughly they were postincubated with 10 mM lysine, 0.3 μCi [^{14}C]lysine/ml, or with MH and then tested for transport in the usual manner. 10 mM lysine is sufficient to induce maximal exchange diffusion (12). Cell water content was determined previously as 1.67 μl/10^6 cells (12).

not monolayers were treated with 0.5% serum, the intracellular lysine levels attained after 15 min preincubation were nearly equal. These internal levels of lysine are sufficient to induce maximal exchange diffusion (12). Nevertheless, after preloading with lysine the initial rate of lysine transport was depressed to the same level by NRS: 76 vs. 73% and 67 vs. 74%. Thus, the depression in lysine transport caused by serum could not be attributed to altered levels of intracellular lysine.

The adenosine system is not subject to exchange diffusion under the conditions employed here.[2] It was also observed that pretreatment of cells with NRS did not affect total radioactivity accumulated after 30 min incubation with 1 mM adenosine.

Separation of Activities.—The factors responsible for LDA and AEA were

[2] Strauss, P. R. Unpublished results.

shown to be different. The former was stable to heating at 100°C for 30 min and lost upon dialysis. The latter was stable to heating for 40 min at 65°C, but not at 100°C; it was nondialyzable. When NRS was passed over Sephadex G25 in MH the elution pattern shown in Fig. 5 was obtained. AEA was excluded by the gel while LDA was included.

Relationship of Transport Factors to Other Substances Known to Affect Macrophages.—Several substances which interact with the macrophage surface were tested for LDA and AEA. One such substance, migration inhibitory factor (MIF), is produced by stimulated lymphocytes (19) and other cells undergoing

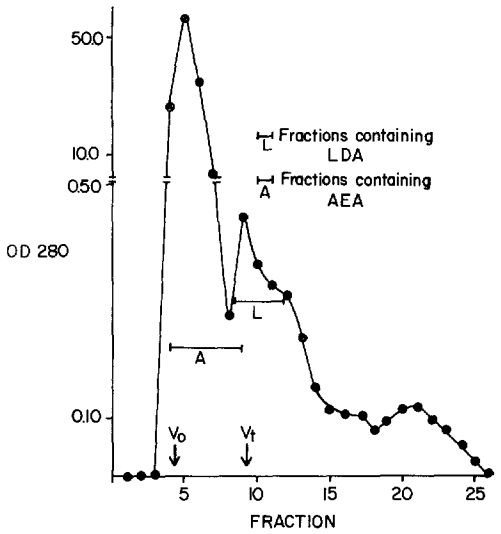

FIG. 5. Fractionation of 25 ml NRS on Sephadex G25. 15-ml fractions were collected from the column. The eluting buffer was MH. Column dimensions were 3.0 × 27.5 cm. V_0 indicates void volume determined by elution of dextran blue (mol wt ca. 10^6). V_t represents the total column volume, determined by elution of ferricyanide. AEA eluted with the leading material while LDA was included but not retained by the column.

cell division (20). A 30 min preincubation with a crude extract containing guinea pig MIF, kindly supplied by Dr. John David, depressed both lysine and adenosine transport. However, the control extract obtained from unstimulated lymphocytes, which lacked MIF, also depressed both transport systems to the same extent. Since guinea pig serum had both LDA and AEA, we think that the species difference was probably not relevant, that lymphocytes may produce substances that affect the transport of nonelectrolytes by macrophages, but that MIF is neither a lysine-depressing factor nor an adenosine-enhancing factor.

Another class of molecules known to interact with the macrophage surface is gamma globulin (gamma G) which binds via its Fc portion to an Fc receptor on the macrophage surface (21–24). Gamma G freed from NRS by precipitation

with ammonium sulfate and subsequent passage over a DEAE-cellulose column (25) maximally and regularly depressed lysine transport by 15% at 100 μg/ml and did not affect adenosine transport. Less gamma G had no demonstrable effect on either transport system. Unstimulated rabbit serum has no more than 10 mg/ml gamma G. Since lysine transport is usually depressed by more than 15% with 0.5% serum and often depressed significantly with 0.05%, which contains no more than 5 μg/ml gamma G, gamma G is probably not responsible for LDA. Moreover, LDA is lost on dialysis; gamma G is not.

Mouse macroglobulin, kindly supplied by Dr. Emil Unanue, was also without activity. However, the lack of activity here might have been due to species differences.

A third class of molecules that interact with the leukocyte surface includes the complement series C3a, C5a, $\overline{C567}$ (26–28), and a fourth, endotoxin (29). We cannot exclude a simple association of complement components with the macrophage surface. However, we can tentatively exclude involvement of the complement cascade because heating at 65°C for 40 min did not affect LDA or AEA. In addition, preincubation of monolayers with 10 μg/ml endotoxin (Difco Laboratories, Detroit, Mich.) for 30 min did not affect transport. With regard to species specificity, human and fetal calf sera had at least the LDA. Guinea pig serum had both LDA and AEA.

DISCUSSION

Two different factors affecting the transport of nonelectrolytes across the macrophage plasma membrane have been demonstrated in NRS. Preincubation for 30 min with the one depresses lysine transport; it is heat stable to 100°C for 30 min and lost upon dialysis. Preincubation for a similar time period with the other enhances adenosine transport; it is sensitive to exposure to 100°C for 30 min and is nondialyzable. The two can be separated by gel filtration on Sephadex G25. Clotting is not required for either activity since plasma has the same amount of activity as serum drawn from the same animal at the same time. The complement cascade is probably not involved since both effects are maintained when serum is heated at 65°C for 40 min. Neither MIF nor rabbit gamma G account for either effect.

Kinetic analyses of how serum affects the adenosine and lysine transport systems reveal greater alterations in V_{max} than in Km. A change in V_{max} can be interpreted as indicating altered numbers of transport sites. However, this remains to be demonstrated.

Several studies concerning the effects of serum on transport by mammalian cells describe uptake over intervals of 5 min to several hours when transport may no longer be rate limiting. In this study the effects of serum components were determined under conditions in which transport was rate limiting, e.g. over 45-s intervals, and which, therefore, involve alterations at the plasma membrane and not mechanisms regulating either accumulation or utilization of substrates.

Nevertheless, two studies may be pertinent to the current work. Wiebel and Baserga (10) showed that 10% fetal calf serum affected the free amino acid pools in human fibroblasts. Within 1 h after a change of serum, free internal leucine and lysine had decreased to about 50% of the control levels. In our experiments, the relative depression of lysine transport was unaltered by preloading with high concentrations of lysine, suggesting that amino acid pools were not depressed by homologous serum. It would be interesting to know whether calf serum was responsible for depressing initial transport rates in human fibroblasts both by decreasing exchange diffusion and by a mechanism similar to that described here, even though protein synthesis was later enhanced in preparation for cell division. However, we note that the calf serum factor was nondialyzable while the rabbit serum factor that affects lysine transport in macrophages described in this report is lost upon dialysis.

Pariser and Cunningham (30) demonstrated antagonistic factors that affect uridine transport by mouse 3T3 cells. One factor is given off by contact-inhibited cells and depresses uridine transport; another from fresh serum which is nondialyzable stimulates uridine transport. While we have no information regarding the affect of NRS on uridine transport, kinetic evidence suggests a common transport system for adenosine and uridine.[3]

Indeed, we do not exclude the possibility of multiple rabbit serum factors, along the lines described by Pariser and Cunningham (30), that affect each of the two transport systems in rabbit lung macrophages. Variability of this kind may account for observed differences in activity from rabbit serum to serum. Stimulatory and inhibitory effects may be closely balanced leading to net stimulation or inhibition.

Lung macrophages are nondividing cells *in situ* (31) and in tissue culture (32). Yet, in this study serum stimulation of nucleoside transport is readily demonstrable. It seems possible that the serum-induced alterations observed in other cells in tissue culture need not be coupled to cell division.

Since it is normal rabbit serum that affects rabbit lung macrophages, one may ask about the relevance of LDA and AEA in vivo. Especially since LDA is dialyzable, it is presumably present in interstitial fluid. Possibly macrophages obtained from lungs are already depressed in lysine transport when we obtain them. Indeed, the surface of the lung contains numerous materials such as surfactant known to affect lung function that may very well alter the physiology of the lung macrophage. In fact, Biggar et al. (33) have reported serum deficiencies in cystic fibrotic patients that affect phagocytosis by macrophages. The mechanisms by which AEA and LDA affect transport and the precise chemical identity of the factors are currently under investigation.

SUMMARY

The effects of normal rabbit serum (NRS) on two transport systems in rabbit lung macrophages have been examined. A 20 min preincubation with serum was

[3] Berlin, R. D. Unpublished results.

required for the effects, which were retained for at least 40 min after serum was removed. No serum was present during the transport studies. (*a*) Preincubation with 0.5 or 1.0% NRS resulted in depression of lysine transport to 59 ± 2.6% (SE, 31 observations) of control levels. The activity was heat stable to 100°C for 30 min and lost after dialysis. Pretreatment with serum did not alter the intracellular concentration of lysine attained when cells were then incubated with 10 mM lysine for 30 min. The relative depression of lysine transport by serum was unaltered by preloading with such high concentrations of lysine. (*b*) Preincubation with 5% NRS resulted in enhancement of adenosine transport by 35 ± 2.3% (SE, 60 observations). Activity was stable to heating at 65°C for 40 min but lost at 100°C for 20 min. It was nondialyzable. Total radioactivity accumulated after 30 min incubation with 1 mM adenosine was unaffected by serum pretreatment. The two activities were separable by passage over Sephadex G25.

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