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THE EFFECTS OF IMMUNOLOGICALLY ACTIVE AMINO ACID AND AMINO SUGAR DERIVATIVES ON TRANSPORT SYSTEMS OF S37 ASCITES TUMOR CELLS[1]

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Abstract. N-α-benzyloxycarbonyl-L-lysine had been shown to possess an immunosuppressive effect, and N-acetyl-D-glucosamine an enhancing effect on the production of IgG and IgM in mice. We studied mechanisms for these effects including a) possible specificity of these agents for cells of the immune system, b) effects of the test compounds on amino acid transport, protein synthesis and/or the rate of growth of some other cellular system. The study system chosen was the mouse S37 ascites tumor cell. Neither compound exerted any effect upon protein synthesis in the S37 cells, or upon survival time of mice bearing the S37 ascites tumor. The effects of the compounds upon antibody production were, therefore, considered to be selective to cells of the immune system rather than general for any actively-metabolizing cells of the mouse. N-α-benzyloxycarbonyl-L-lysine did exhibit selective affinity for the L and A amino acid transport systems of the S37 cells. This effect modified previous concepts of the specificities of the two principal neutral amino acid transport systems.

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We had found that low molecular weight amino acid and amino sugar derivatives were capable of influencing the humoral immune response of mice at dose levels as low as 10 mg/kg (Lewis *et al* 1978, 1974). N-acetyl-D-glucosamine (NAG) the sugar moiety common to many glycoproteins and immunoglobulins, N-α-benzyloxycarbonyl-L-lysine (Cbz-Lys), a synthetic amino acid derivative, and L-lysine, the naturally-occurring amino acid (Sigma Chem. analytically pure) served as chemical precursors and possible metabolites for a series of synthetic N-glycosyl amino acids designed to influence the immune response (Lewis *et al* 1974). Cbz-Lys proved to be a potent immunosuppressant; NAG demonstrated immuno-enhancing properties at higher doses; L-lysine had no observable effect upon the humoral immune response. The biological activity of small peptides capable of inhibiting protein synthesis has been established in the case of antibiotics (Waksman 1960) and considerable evidence has accumulated regarding the specific chemical characteristics of antigenic molecules derived from amino acids (Day 1972). We observed that the most prominent effects on the immunological system of test mice challenged with the antigen sheep red blood cell occurred only when test compounds were administered during the later stages of the immune response. In an effort to establish whether our observations were due to specific effects upon cells of the immune system, or whether these compounds might exert general effects upon other cells actively engaged in protein synthesis, we studied their effects upon amino acid transport, protein synthesis, and growth of S37 ascites tumor cells, as judged by survival experiments.

MATERIALS AND METHODS

Methods of S37 cell propagation and amino acid transport experiments were in general as reported earlier (Matthews *et al* 1969, 1972). Low and high concentration uptake of radiolabeled amino acids were measured for 2-minutes at 20 °C. To discriminate between amino acid transport systems L and A, uptake of 0.1 mM ^{3}H-L-histidine in the presence of 20

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mM N methyl α amino isobutyric acid was used as a measure of system L activity and uptake of 10 mM ^{3}H-L-histidine in the presence of 15 mM BCH (exo-2-amino-bicyclo [2,2,1]-heptane-2-carboxylic acid) (Matthews *et al* 1975) was used as a measure of sytem A activity. Previous results suggested that the observed values for system L activity would be 98% test uptake while that given for system A would be greater than 95% test uptake (Matthews *et al* 1975). Test compounds were used at a concentration of 20 mM and reported values represent averages of a minimum of 3 samples, each experiment being performed at least 3 times.

In experiments in which it was desirable to measure incorporation of labeled amino acid (0.125 mM L-histidine or L-lysine) into cell protein as well as the soluble amino acid fraction, incubations were carried out at 37 °C for 3 hr followed by disruption by 95% ethanol for 15–30 min (Matthews *et al* 1972, 1975). The ethanolic extracts were cleared of cell solids by centrifugation at 1940 x g for 1 min. Aliquots of 0.5 ml were taken from the individual 5 ml ethanolic extracts for the determination of soluble amino acid label by liquid scintillation counting. The remainder of the ethanol was siphoned off, taking care not to disturb the cell solids and individual cell pellets were resuspended in 95% ethanol. Centrifugation was repeated, ethanol was again siphoned off, at which time 1 ml of TS-1 tissue solubilizer (Research Products International) was added to each pellet. Cell solids were then disrupted with a glass rod, samples were warmed in a 37 °C water bath to speed dissolution and 6 ml of a liquid scintillator was added to each tube before transferring the sample to a counting vial. Tubes were rinsed into counting vials with a second 6 ml of liquid scintillator.

For studies on survival times of mice bearing the ascites tumor, test and control animals (n=10) were injected with 0.3 ml of approximately 1:10 suspension of S37 cells on day one. On day 4, mice were matched by appearance and divided randomly into test and control groups. Control animals received injections of 0.5 ml Krebs-Ringer-phosphate while test animals received injections of 0.5 ml test solution in sterile distilled water on days 5 to 8. Animals were monitored for survival times and the mean survival computed plus or minus standard deviation of observations.

RESULTS

Cbz-Lys, a potent inhibitor of immunoglobulin synthesis *in vivo*, appeared to have no significant activity *in vitro* as an inhibitor of amino-acid retention or protein synthesis in the mouse ascites cell (table 1). NAG, an enhancer of immunoglobulin synthesis *in vivo*, also failed to exert any significant effect on these parameters in mouse ascites cells. Mean survival time in days for the control group was 9.30±0.45 days and for the experimental group 9.40±0.65. Hence, Cbz-Lys did not appear to affect S37 cell growth as judged by mouse survival.

TABLE 1

Inhibition of Steady-State Amino Acid Retention and Protein Synthesis with N-α-Benzyloxycarbonyl-L-Lysine (Cbz-Lys), N-Acetyl Glucosamine (NAG) and L-Lysine.

A. Effects on ^{3}H-L-histidine retention and incorporation.*

Compound Competing	Soluble Amino Acid Label (% control)	Solid Label (% control)
^{3}H-L-histidine control	100	100
Cbz-Lys	100±10	90±7
NAG	93± 9	96±6
L-lysine	101± 5	94±5

B. Effects on ^{3}H-L-lysine retention and incorporation.*

^{3}H-L-lysine control	100	100
Cbz-Lys	104± 6	99±5
NAG	98± 5	93±6
L-lysine	108± 6	66±6

*All values represent mean plus or minus SEM of 4 separate experiments utilizing triplicate samples. Experiments were carried out at 37 °C for 2 hr in the presence of 0.125 mM ^{3}H-L-histidine or ^{3}H-L-lysine, and test compounds were examined at 5.0 mM.

The effects of Cbz-Lys on initial velocity of ^{3}H-histidine uptake showed moderate interactions with both transport systems L and A (table 2). NAG had a more marginal effect. In view of the differences of these compounds from the usual range of substrates examined, the observed effects upon transport were indeed surprising and worthy of note.

DISCUSSION

Lewis *et al* (1978) had demonstrated that synthetic agents based upon the components NAG, L-lysine and Cbz-Lys were capable of influencing the immune response at low dose levels. Enhancement or depression of splenic plaque forming cells (PFC) for IgM and IgG were observed depending on the assay time and treatment interval relative to the administration of suitable antigenic substances (Jerne *et al* 1963). NAG had a stimulatory effect on PFC.

TABLE 2

*Effects of N-α-Benzyloxycarbonyl-L-Lysine (Cbz-Lys) and N-Acetyl Glucosamine (NAG) on ^{3}H-L-Histidine Uptake Activities of Transport Systems of S37-ascites Tumor Cells.**

Competing Compound	Activity of System L** (% control)	Activity of System A*** (% control)
10 mM sucrose (control)	100	100
10 mM Cbz-Lys	70±1	77±1
10 mM NAG	86±5	83±5

*All data are mean plus or minus SEM of 3 runs on triplicate samples.

**L-system values were determined utilizing 0.1 mM ^{3}H-L-histidine in the presence of 20 mM N-methyl-α-amino isobutyric acid.

***A-system values were determined utilizing 10 mM ^{3}H-L-histidine in the presence of 15 mM exo-2-amino-bicyclo [2,2,1]-heptane-2-carboxylic acid.

Cbz-Lys had demonstrated a decrease in PFC/10^6 spleen cells while L-lysine showed no increase relative to control animals. These differences had been noted only when test compounds were administered during the later stages of the immune response. The absence of effects on *in vitro* protein synthesis, amino acid retention, or growth of mouse S37 ascites tumor cells observed in our study imply that the immunological results reported previously were specific to cells of the immune system rather than general responses for any cell system actively synthesizing protein.

A major problem in the area of amino acid transport is the differentiation of contributions of multiple specificities. The existence of such multiple systems for neutral amino acids in ascites tumor cells has been known since the work of Tenenhouse *et al* (1960). One of the approaches used in the differentiation of amino acid transport systems has been variation of the structure of an amino acid or analog, and the use of such compounds either as test substrates or as competitive inhibitors to restrict the number of transport systems being employed (Matthews *et al* 1975, Christensen *et al* 1965). This approach has also given some information regarding the nature of substrate-transport system interaction. It has previously been shown that the carboxyl group, one point of attachment of the substrate to transport systems L and A, can be varied to a methyl ketone group or a methyl ester group so that a negatively charged moiety is not needed for interaction to occur (Mathews *et al* 1975). Christensen and coworkers (1965) had suggested that N-methylation of amino acids obliterated any interaction with system L but did not impair interaction with system A. We had confirmed this observation in the case of N-methyl-alpha-amino-isobutyric acid and had observed that proline exhibited affinity only for system A (Matthews 1972, 1975). These findings suggested that the α-amino group, a second point of attachment of the substrate to the transport system, was probably an unsubstituted primary amine. For interaction with system L to occur, this amine would bear a positive charge at neutral pH. Cbz-Lys not only has a substituent attached to the α-amino group, but it carries a substituent that removes the basic character of the α-amino group. The present result suggests that the specificity requirement of system L relative to the α-amino group may not be narrowly defined as has previously been suggested.

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