The Effect of Hexosamine Inhibitors on the Secretion of γ-Globulin

Secretion is the final step in the production of humoral antibody. Although attachment of the carbohydrate moiety to nascent polypeptide chains precedes the extracellular transport of antibody[1], the necessity for such attachment and the role of the carbohydrate piece in the transport mechanism is still unclear. This report describes an attempt to interrupt the attachment of the carbohydrate moiety by an hexosamine inhibitor and to assay for γ-globulin in the extracellular fluid of an in vitro system.

Cell suspensions were prepared from rabbit lymph nodes or chicken spleens. The rabbits were injected with complete Freund's adjuvant seven days prior to sacrifice; the chickens were stimulated with sheep red blood cells three days prior to use. The tissues were collected, minced, and pressed through a No. 60 mesh stainless steel screen. Cell suspensions were prepared in L-leucine, L-glutamine-free minimal essential medium without antibiotics and adjusted to a final concentration of 10×16^6 cells/ml. Cultures of 2.5 ml were incubated in 35×10 mm siliconized petri dishes in an atmosphere of humid 5% CO_2 at 37 °C.

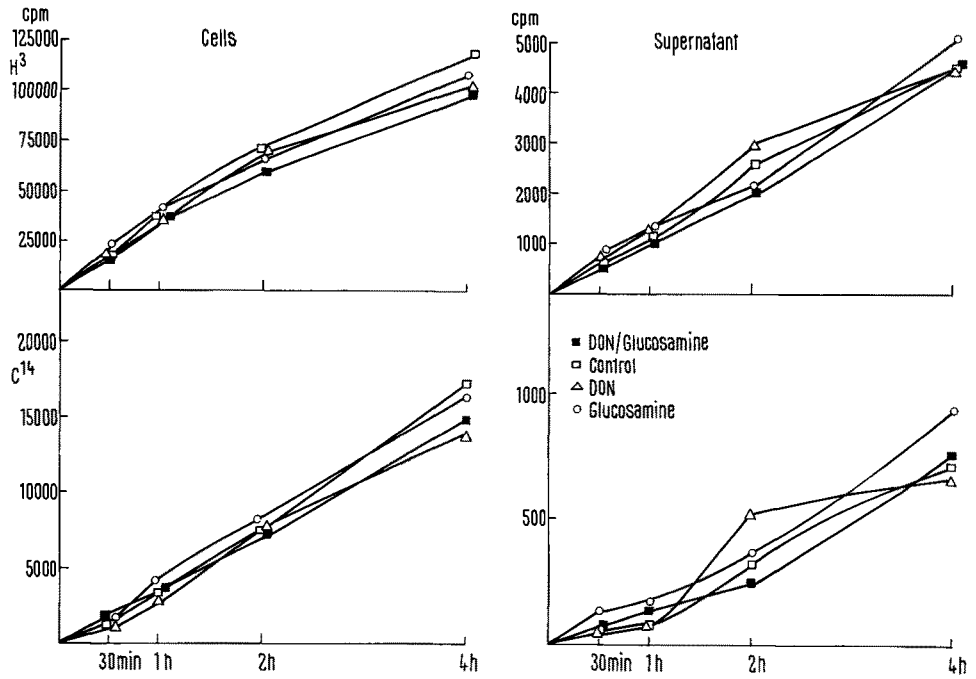
An hexosamine inhibitor, 6-diazo-5-oxonorleucine (DON), duazomycin A or duazomycin B, was added to the cell cultures in concentrations ranging from 15 μg/ml to 150 μg/ml. Control cultures were set up with the following additives: Dulbecco's phosphate buffered saline, glucosamine 1 mg/ml (adjusted to pH 7.4), and inhibitor plus glucosamine 1 mg/ml. All cultures were incubated for 15 min prior to the addition of radioactive label.

Paired cultures were labelled with 25 μc of 4,5 ^{3}H-L-Leucine (specific activity 6 c/mM) or 20 μc of ^{14}C-1-acetic acid (specific activity 13.7 mc/mM) for varying periods. At the termination of the pulse the tubes were centrifuged at $700 \times g$, 4 °C and the cells and supernatant separated and frozen.

Glycoprotein was extracted by boiling cell pellets in a waterbath for 3 min and washing twice with 6% trichloroacetic acid and 0.5% phosphotungstic acid; supernatants were washed directly upon thawing. This was followed by two washes of acetone (to remove lipid contaminants), two of ethanol-ether (2:1), and one of ether. The precipitates were dried at 40 °C, dissolved in 0.2 ml of 1N NaOH, heated to 100 °C for 1 min and 0.3 ml of distilled water added. A 0.1 ml aliquot was dissolved in 0.3 ml Nuclear Chicago Solubilizer. The radioactivity of each sample in 10 ml of scintillation fluid was measured in a Packard Tri-carb liquid scintillation counter. The presence of specifically co-precipitable extracellular γ-globulin was determined according to the method of ALM and PETERSON[2].

No significant difference was found between treated and control cultures on measurement of ^{3}H-leucine and ^{14}C-acetate in cells and supernatant at varying time periods (Figure). Absence of an effect was also found in experiments (Table) in which a 4-h pulse and 2 widely disparate concentrations of inhibitor were used. The co-precipitation values also demonstrated no differences in uptake of radioactive label except when exceedingly high concentrations of inhibitor were added. However, under these conditions, reduction in uptake of C^{14}-acetate with 150 μg/ml of DON was not reversed by the addition of glucosamine.

The hexosamine inhibitors prevent de novo synthesis of glucosamine derivatives through inhibition of transamidation[3,4], and reversal of this effect should be possible by the addition of free glucosamine[4]. Since available evidence indicates that the carbohydrate moiety of the γ-globulin molecule is attached via an N-acetyl glucosamine prior to secretion[5,6] it was anticipated that incubation of globulin-producing cells with an hexosamine inhibitor might prevent globulin secretion. By labelling both the polypeptide (^{3}H-leucine) and the carbohydrate



^{3}H-leucine and ^{14}C-acetate incorporation in chicken spleen cell cultures pulsed for varying time periods. cpm, counts per minute; C, saline control; D, DON 30 μg/ml; G, glucosamine 1 mg/ml; D/G, DON 30 μg/ml + glucosamine 1 mg/ml.

(^{14}C-acetate) portions of the γ-globulin molecule and assaying for specifically co-precipitable material in the extracellular fluid, it might be possible to determine whether secretion had been prevented by blocking attachment of the carbohydrate.

Our results indicate that at concentrations comparable to those effective in other cell systems[4], hexosamine inhibitors, under these experimental conditions, do not alter the secretion of γ-globulin. Only very high concentrations appear to reduce the amount of specifically co-precipitable carbohydrate without affecting the amount of labelled protein present in the extracellular fluid. This would imply that some γ-globulin was secreted without attached carbohydrate. We cannot exclude the possibility that the ^{14}C-acetate was incorporated into substances other than γ-globulin which co-precipitated under the present conditions.

2.5 ml cultures of 25×10^6 chicken spleen cells	H^3-leucine (cpm)		C^{14}-acetate (cpm)	
	Total	Co-precipitation	Total	Co-precipitation
Control	4769	2928	710	243
DON 30 µg/ml	5229	2996	798	233
Glucosamine 1 mg/ml	4609	2867	727	264
DON 30 µg/ml Glucosamine 1 mg/ml	4926	2501	745	273
Control	5076	3724	740	267
DON 150 µg/ml	4962	3233	514	118
Glucosamine 1 mg/ml	4701	3413	672	211
DON 150 µg/ml Glucosamine 1 mg/ml	4270	2744	503	105

cpm, counts per minute. The values in this representative experiment showing total glycoprotein and co-precipitable material in extracellular fluid are the means of duplicate cultures.

Zusammenfassung. Hemmung von Hexosamin vermag die Sekretion von γ-Globulin durch antikörperproduzierende Zellen unter in vitro Bedingungen nicht zu verhindern. Dies könnte darauf hinweisen, dass unter den gewählten Bedingungen kohlehydratfreie γ-Globulinmoleküle sezerniert werden.

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Extent of Immunoglobulin Light Chains Heterogeneity Revealed by Isoelectric Focusing

The light polypeptide chains, prepared from normal immunoglobulins of a variety of species, form 8–10 electrophoretic bands on starch or acrylamide gels in urea containing buffers[1]. Each electrophoretic band seems to contain a certain number of different kinds of L-chains, at least 5 as calculated by CHOULES and SINGER[2], so there appear to be 40–50 distinct forms of light chains[3].

In order to prove experimentally that each band consists of a number of components having the same total electric charge under the condition of electrophoresis, we resorted to the isoelectric focusing method (IEF)[4]. This method allows in principle the focusing of L-chains into as many bands as there are kinds of L-chains with distinct isoelectric points. IEF turned out to be efficient at the advanced fractionation of immunoglobulins[5] but it was not found significant in Bence-Jones (B-J) proteins and light chains analyses[6].

We submitted to both IEF and electrophoresis on acrylamide gels containing urea, light chains isolated from rabbit immunoglobulin G (IgG) and from a human IgG myeloma and an urinary B-J protein.

Materials and methods. Rabbit and human myeloma IgG were prepared by DEAE-cellulose chromatography[7]. Light chains were isolated by the method of FLEISCHMAN et al.[8]. The B-J protein(λ-type) was isolated from the urine of an IgD myeloma patient by precipitation with ammonium sulphate and subsequent gel filtration on a column of Sephadex G-75 eluted with 0.1 M phosphate buffer pH 7.5 containing 6 M urea. The first peak contains the B-J protein as dimer and the second peak the monomer. The other chromatographic peaks contain the urinary proteins.

Electrophoresis was carried out by a horizontal technique in 6% acrylamide gels made 6 M in urea[9]. The slides length was 8 cm and the gel thickness was 1.5–3 mm. Electrophoresis was performed for 8 h for the rabbit L-chain and 4 h for the L-myeloma chain and urinary B-J protein, at a potential gradient of 2 V/cm.

The protein extraction from some electrophoretic bands was made by an electro-elution technique similar to that described by LEWIS and CLARK[10]. The bands were preliminarily localized by rapid staining of a strip cut from the length of the gel. The protein eluted from the

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