Vol. 96, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1980 Pages 211-218

EFFECT OF CONCANAVALIN A ON GANGLIOSIDE METABOLISM OF HUMAN LYMPHOCYTES

ALLAN J. YATES AND SARITA L. MATTISON
Department of Pathology

RONALD L. WHISLER
Department of Medicine

College of Medicine, Ohio State University
Columbus, Ohio, 43210

Received July 28,1980

SUMMARY

The effect of Con-A on the incorporation of radioactivity from [^{14}C]-glucosamine into gangliosides of human lymphocytes was investigated. Compared with non-stimulated lymphocytes there was increased incorporation into gangliosides and total lipids within the first 24 hours of exposure to Con-A. Ganglioside synthesis also occurred in later time intervals within the 96 hour incubation period. GM3 accounted for 80% of the labeled ganglioside in Con-A stimulated cells at all times studied. Thus ganglioside synthesis is not only associated with cellular division, but also occurs within a few hours of lymphocyte activation representing an extremely early prereplicative event.

Several recent studies indicate that gangliosides might be important in the lymphocyte interactions required for normal immune homeostasis and immune reactivity (1-4). Additionally, others have suggested that alterations of exogenous gangliosides may contribute to aberrant immunoregulation in certain disease states (5). For example, exogenous gangliosides are bound by human peripheral blood mononuclear cells (PBMC) and impair lymphocyte activation by nonspecific mitogens and allogeneic stimuli (6,7). More recently, gangliosides have been identified as specific surface markers for distinct functional lymphocyte populations and quite possibly may function as lymphocyte membrane receptors (8,9). Therefore, it has become important to investigate ganglioside metabolism in immune reactive cells. Glucosamine is an aminosugar which is one of the precursors of sialic acid, the characteristic constituent of

Abbreviations: Con-A, Concanavalin-A; PBMC, peripheral blood mononuclear cells; S.I., stimulation index; PHA, phytohemagglutinen; NeuNglc, N-Glycolyl neuraminic acid; NeuNac, N-acetyl neuraminic acid; GM3, hematoside

This work was supported by American Cancer Society Grant IM-199.

0006-291X/80/170211-08$01.00/0

gangliosides (10). We report here the effects of Concanavalin-A (Con-A) on the incorporation of radiolabel from [^{14}C]-glucosamine into the ganglioside of human PBMC.

MATERIALS AND METHODS

Immunological - PBMC were separated from heparinized human blood by centrifugation over Ficoll-hypague gradients (11). The cells were washed three times with RPMI 1640 and resuspended at 1.7 x 10^6 cells/ml in RPMI 1640 supplemented with 12% fetal bovine serum (Lot No. 93481, Microbiological Associates, Walkersville, M.D.). The PBMC were stimulated with an optimal concentration (18 ug/ml) of Concanavalin A (Wellcome, Beckenbaum, England) as previously described (12) in 25 cm^2 flasks (No. 3013, Corning Inc., Corning, New York). To assess proliferative responses, 1 µCi of [^{3}H] thymidine (New England Nuclear, Boston, Mass.) was added to 1.7 x 10^5 PBMC eighteen hours prior to processing on glass wool filter pads. At the specified times, the incorporated radioactivity was determined by suspending the filters in Aquasol II scintillation fluid and counting in a beta scintillation counter. The variation of [^{3}H] thymidine incorporation between individual samples did not exceed 20 percent.

Biochemical - Lymphocyte pellets were transferred to size BB Thomas tissue grinders, 0.5 gm normal human brain white matter added as carrier tissue, and the gangliosides extracted and partitioned according to Suzuki (13) with an additional 3 washes of theoretical upper phase containing water. The pooled upper phases were treated with E. coli alkaline phosphatase (EC 3.1.3.1) and snake venom phosphodiesterase (EC 3.1.4.1) (Worthington Biochemical Corp.), then subjected to alkaline methanolysis (14,15), and dialyzed overnight against several distilled water changes. The dialysate was taken to dryness and the gangliosides purified by silicic acid column chromatography (16). Radioactivity in aliquots of ganglioside samples and lower phase (10% of total) were counted in Aquasol II (New England Nuclear) on a Beckman LS 8000 liquid scintillation counter. When a sufficient number of counts was available, the remainder was applied to a silica gel 60 (Merck) thin layer plate with both unlabeled normal human cerebral cortex gangliosides and radiolabeled rat brain gangliosides (10) as standards, and the plate developed in the solvent chloroform:methanol:0.2% $CaCl_2 \cdot 2H_2O$ (60/40/10). The plate was air dried, Kodak XR-2 film applied over the gel and placed in the dark for 12 weeks. The lanes with the human brain ganglioside standards were sprayed with resorcinol reagent allowing the visible spots on the developed film to be identified by matching them with these standards. The gel containing each radiolabeled ganglioside, as well as areas between, above, and below visible bands, were scraped into separate scintillation vials with Aquasol II and radioactivity counted.

RESULTS

Both control and Con-A stimulated cultures incorporated radioactivity into gangliosides and lower phase (non-ganglioside) lipids (Table I). Most cultures incorporated 2 to 4 times as much radioactivity into the lower phase lipids as into gangliosides. Dividing the Con-A stimulated by the control values gives a stimulation index (S.I.). The average percentage error between

TABLE I

EFFECTS OF CON-A ON [^{14}C]-INCORPORATION INTO TOTAL LIPIDS AND GANGLIOSIDES

LABELING INTERVAL[a]	GANGLIOSIDES			TOTAL LIPID		
	Control[b]	Con-A[b]		Control[b]	Con-A[b]	
0-6	173	165	(0.95)	252	356	(1.41)
6-18	160	242	(1.51)	307	525	(1.71)
18-24	139	206	(1.48)	191	346	(1.81)
0-24	187	369	(1.97)	556	927	(1.67)
24-72	161	668	(4.14)	514	4664	(9.07)
72-96	152	368	(2.42)	523	1267	(2.42)
0-96	181	1651	(9.12)	614	5063	(8.24)

a. Time interval in hours following Con-A stimulation during which [^{14}C]-1-D glucosamine was added.
b. Results expressed as counts per minute X 10^{-1} of radioactivity per 10^{6} cultured cells are means of duplicate samples. Number in parenthesis is the stimulation index, i.e. the value for Con-A stimulated culture divided by value for control culture.

duplicate samples was 17% which indicates that a S.I. of 1.4 or less could occur as the result of experimental error. Therefore, only those S.I. greater than 1.4 are considered significant.

No significant stimulation of ganglioside synthesis in response to Con-A was seen within the first 6 hours (S.I.=0.95). However, subsequent to this all ganglioside stimulation indices were 1.48 or greater. Similarly, an S.I. of 1.41 occurred within the first 6 hours for lower phase lipid values, but all those subsequently were 1.7 or greater.

Only those Con-A stimulated cultures exposed to [^{14}C]-glucosamine for at least 24 hours incorporated sufficient radioactivity for thin layer chromatographic analysis. Table II shows the percentage distributions of radioactivity recovered from the plates which co-chromatographed with the standard gangliosides in duplicate experiments. Note that at all times studied, the major radiolabeled gangliosides is GM3 with no other gangliosides having over 6% of the total.

TABLE II

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY AMONG GANGLIOSIDES OF CELLS STIMULATED WITH CONCANAVALIN-A[a]

LABELING INTERVAL[b]	GANGLIOSIDE[c]				
	GM3	GM2	GM1	GD3	OTHER[d]
0-24	72.5; 89.4	7.2; 0.0	4.1; 7.8	7.2; 2.8	9.0; 0.0
24-72	84.5; 94.6	3.4; 1.7	3.5; 2.9	4.8; 0.8	3.7; 0.0
72-96	79.2; 83.7	3.4; 3.2	2.4; 13.1	8.7; 0.0	6.4; 0.0
0-96	89.4; 90.0	3.3; 2.1	2.1; 2.0	4.4; 5.9	0.7; 0.0

a. Results are of duplicate experiments for each time interval.
b. Same as Table I-a.
c. Ganglioside nomenclature is that of Svennerholm (22).
d. Contains data from gels taken from the same level as the standards GT_1, GD1b, GD1a and GD-2.

The data presented in Table III demonstrates that no significantly increased [^{3}H]-thymidine incorporation occurred prior to 72 hours of incubation.

DISCUSSION

These results indicate that Con-A augments the incorporation of radiolabel from [^{14}C]-glucosamine into gangliosides of human PBMC, and that this begins within the first 18 hours of stimulation. This is of particular interest because accelerated incorporation of thymidine into these cells does not occur until 72 to 96 hours following stimulation by Con-A (Table III). Therefore, the increased radiolabeling of gangliosides is an early, prereplicative phenomenon. Increased labeling of the non-ganglioside total lipid fraction

TABLE III

[^{3}H]-THYMIDINE INCORPORATION INTO PBMC[a]

	DURATION OF CULTURE (HOURS)				
	6	18	24	72	96
Control	211±10	494±107	658±31	1169±285	574±134
Con-A[b]	202±19	488±53	668±141	77,501±3839	61,189±7475

a. Results are expressed as mean counts per minute ± S.D. for triplicate cultures.
b. Responses of cultures stimulated with 18 ug/ml Con-A.

also occurred with a similar time course in response to Con-A. Although further characterization of the labeled constituents in this fraction was not done, it is likely that much of the radioactivity is in glycolipids.

While the early labeling of gangliosides and other lipids is interesting, it is not the only time period during which augmented labeling of these constituents occurred. Indeed, the greatest stimulation index occurred between 24 and 72 hours (with the exception of the S.I. for the entire 96 hours studied). The exact meaning of this is obscure, but it indicates that Con-A may stimulate the synthesis of gangliosides and neutral glycolipids independent of its effects on DNA synthesis.

Augmentation of lipid synthesis due to lectin stimulation has been noted previously. Kay (17) demonstrated that PHA causes a rapid increase in the rates of incorporation of several precursors into lipids of human lymphocytes which begins within the first hour of exposure to the lectin. Subsequently, it was found that one of the earliest metabolic responses of lymphocytes to PHA is a large increase in the rate of incorporation of $[^{32}P]O_4^{=}$ into phosphatidyl inositol (18). Employing mouse thymocytes, Rosenfelder et al. (19) found that there is increased radioactivity incorporated into both neutral phospholipids and gangliosides from radiolabeled galactose, serine, and glucosamine in response to Con-A. Although there was increased labeling of the phospholipids both early and late, neutral glycolipids and gangliosides only became heavily labeled at the later times for these cells (30 hours post stimulation). Inouye et al. (20) found that PHA augmented the incorporation of $[^{3}H]$-glucose into lipids of human lymphocytes but most of the radiolabel was in neutral lipids. A similar effect was seen using $[^{3}H]$-galactose as precursor. However, over 60% of tritium in lipid of these cells was in polar lipids and more than 40% was in glycolipids. Ceramide trihexoside was the most radiolabeled glycolipid, but incorporation into glycosylceramide, ceramide dihexoside, and globoside was also accelerated.

Narasimhan et al. (21) found that Con-A stimulation of sheep PBMC increased the incorporation of radiolabel from [^{14}C]-galactose into both the neutral glycolipid and ganglioside fractions. Triglycosylceramide was the most heavily labeled neutral phospholipid and incorporation into diglycosylceramide was also increased. However, the radioactivity present in monoglycosylceramide was actually lower in Con-A stimulated cells. When [^{14}C]-galactose was present for the first 24 hours of exposure to Con-A, the major labeled ganglioside was the NeuNglc containing GM3. However, the NeuNac GM3 was most heavily labeled both following 72 hours of exposure to Con-A and [^{14}C]-galactose, and when [^{14}C]-galactose was added during the time interval 48 to 72 hours following Con-A stimulation.

In our studies, GM3 of Con-A stimulated cells contained over 70% of all the radioactivity incorporated into gangliosides (Table II). Furthermore, there was no alteration of chromatographic mobility of radiolabeled GM3 to suggest a change in its sialic acid constituents (Figure 1). While the

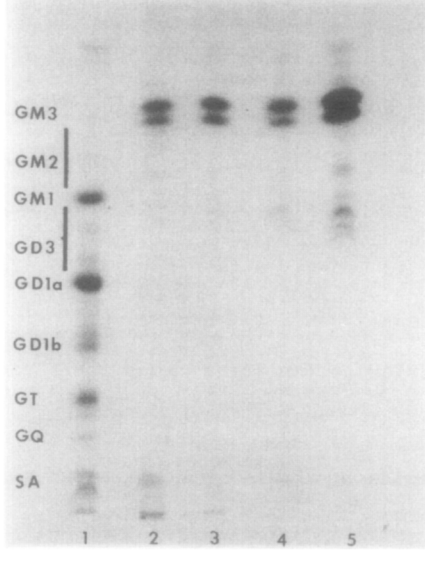

Figure 1. Autoradiogram of radiolabeled gangliosides separated on silica gel-60 thin layer plate in chloroform-methanol-0.2% $CaCl_2 \cdot 2H_2O$ (60:40:10). Lane 1 - 12 day old rat brain ganglioside standards labeled by intracranial injection of [^{14}C]-glucosamine (14). All other lanes are gangliosides from Con-A stimulated human PBMC exposed to [^{14}C]-glucosamine during the following time intervals: Lane 2, 0-24 hours; Lane 3, 24-72 hours; Lane 4, 72-96 hours; Lane 5, 0-96 hours. System of ganglioside nomenclature is that of Svennerholm (22). SA-free sialic acid.

exact significance of this is unknown, it is interesting in view of two of our previous observations: (a) that GM3 is one of the least inhibitory gangliosides to Con-A stimulated lymphoblastic transformation; (b) that there is a limited capacity for ganglioside binding to PBMC (6,7). This raises the possibility that prior to commitment to cell division, the cell membrane may shed or displace inhibitory gangliosides and replace them with inactive constituents like GM3.

As suggested by Narasimhan et al. (21) both their results and ours could be explained at least partially on the basis of an increased transport rate of radiolabeled precursor by Con-A stimulated cells. Therefore, it will be of considerable interest to examine the activities of biosynthetic enzymes responsible for ganglioside synthesis in these cells under similar conditions as we have studied here. Now that there is substantial evidence implicating gangliosides as immunoregulatory compounds, it is important to characterize alterations in their metabolism during various phases of lymphocyte activation.

REFERENCES

1. Esselman, W.J., and Miller, H.C. (1977) J. Immunol. 119, 1994-2000.
2. Miller, H.C., and Esselman, W.J. (1975) J. Immunol. 115, 839-843.
3. Ryan, J.L., and Shinitzky, M. (1979) Eur. J. Immunol. 9, 171-175.
4. Sela, B.A. Cell Immunol. 49, 196-201.
5. Lengle, E.E., Krishnaraj, R., and Kemp, R.G. (1979) Cancer Res. 39, 817-822.
6. Yates, A.J., Hitchcock, C.L., Stewart, S.S., and Whisler, R.L. (1980) American Chem. Soc. Symposium Series - in press.
7. Whisler, R.L., and Yates, A.J. (1980) J. Immunol. - in press.
8. Speigel, S., David, A., and Wilchek, M. (1979) Proc. Natl. Acad. Sci (USA) 76, 5277-5281.
9. Young, W.W., Hakomori, S.I., Durdik, J.M., and Henney, C.S. (1980) J. Immunol. 124, 199-201.
10. Burton, R.M., Garcia-Bunuel, L., Golden, M., and Balfour, Y.M. (1963) Biochem. 2, 580-585.
11. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97), 77-89.
12. Stobo, J.D., Paul, S., VanScoy, R.E., and Hermans, P.R. (1976) J. Clin. Invest. 57, 319-328.
13. Suzuki, K. (1965) J. Neurochem. 12, 629-638.
14. Kanfer, J.N. (1969) Methods in Enzym. XIV, 660-664.
15. Dawson, G., Kemp, S.F., Stoolmiller, A.C., and Dorfman, A. (1972) J. Lipid Res. 13, 207-219.
16. Yates, A.J., and Wherrett, J.R. (1974) J. Neurochem. 23, 993-1003.

17. Kay, J.E. (1968) Nature 219, 172-173.
18. Wedner, H.J., and Parker, C.W. (1976) Prog. Allergy 20, 195-300.
19. Rosenfelder, G., Van Eyk, R.V.W., Manner, P.A., and Muhbradt, P.F. (1978) 83, 571-580.
20. Inouye, Y., Handa, S., and Osawa, T. (1974) J. Biochem. 76, 791-799.
21. Narasimhan, R., Hay, J.B., Greaves, M.F., and Murray, R.K. (1976) Biochim. Biophys. Acta 431, 578-591.
22. Svennerholm, L. (1970) Comprehensive Biochemistry, edit: Florkin M. and Stotz, E., pp. 201-227, Elsevier Publishing, New York.