RAPID, PERIODATE-INDUCED STIMULATION OF PERMEABILITY AND MACROMOLECULAR SYNTHESIS IN CHICKEN ERYTHROCYTES

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SUMMARY: Brief treatment of chicken erythrocytes with periodate induces a rapid increase in uptake of ^{3}H-uridine and its incorporation into RNA. Incorporation but not uptake is inhibited by Actinomycin D, but neither parameter is affected by ethidium bromide. Thymidine uptake is unaltered; there is neither hemolysis nor altered permeability to trypan blue. The rapid responses are similar in magnitude to those elicited by biological mitogens from lymphocytes. We interpret these results to mean that avian erythrocytes can respond in a normal way to signals like those active on lymphocytes and hence are a useful system to study mechanisms whereby signals at the cell periphery activate nuclear function.

The transformation of resting lymphocytes into large blast cells capable of several mitoses has been well described and documented for a variety of stimuli (mitogens) purified from biological sources. These include bacterial products such as the lipopolysaccharides (1), antibodies (2), and plant lectins (3), most notably PHA*. More recently it became known that exposure to sodium periodate also enhances a delayed synthesis of DNA in lymphocyte populations (4-6). The chemistry of periodate oxidation is defined, and its bulk target in the human erythrocyte is known to be the sialic acid of the major sialoglycoprotein of the plasma membrane (7); this system is less complex to analyse than the transformation phenomenon associated with PHA. Novogrodsky and Katchalski have reported delayed stimulation of thymidine incorporation into DNA after sequential treatment of cells with neuraminidase and galactose-oxidase, thus focusing attention on the role of the chemically reactive aldehyde group generated by each method (8).

With lymphocytes, the earliest measurable effects, evident within minutes after lectin addition, are changes in membrane properties as manifested by the increased uptake of nutrients (8-11) and ions from the medium (12,13), the turnover of membrane components (14-18), and the activity of several membrane-bound enzymes (19-21). Almost equally rapid are metabolic responses within the nucleus, such as increased rates of histone acetylation (22) and activities of RNA polymerases (23).

In attempting to clarify mechanisms whereby signals at the cell surface activate nuclear functions, we have employed the chicken erythrocyte (CRBC*) as target cell and sodium periodate as stimulus. CRBC's contain nuclei with condensed and biosynthetically dormant chromatin, a few mitochondria, but--unlike

*Abbreviations: PBS - phosphate-buffered saline (26); PHA - phytohemagglutinin; CRBC - chicken red blood cells; MEM - minimal essential medium.

Table I

Dependence of ^{3}H-Uridine Metabolism on Periodate Concentration[1]

mM IO_4	Uptake cpm/µg	Uptake IO_4/Control	Incorporation cpm/µg	Incorporation IO_4/Control
Control	312	1.0	33	1.0
0.1	321	1.0	37	1.1
0.3	479	1.5	104	3.1
0.5	595	1.9	129	3.9
0.75[2]	383	1.2	40	1.2
Control	212	1.0	17.2	1.0
PHA	277	1.3	35.4	2.1

[1]Samples were labeled for 3 hrs with 5µc/ml ^{3}H-uridine at 200 picamoles/ml. The PHA data represent another bleeding of the same bird; 100µg/ml PHA was added for the entire incubation period. Uptake represents cpm of cold perchloric acid soluble material/µg RNA. Incorporation represents cpm acid-precipitable, alkali labile material/µg RNA.

[2]Some hemolysis at 3 hrs after resuspension in PBS. No hemolysis upon resuspension in PBS plus 15% fetal calf-serum.

lymphocytes--lack polyribosomes for protein synthesis (24). Following Sendai-virus induced fusion with actively metabolizing cells, CRBC's can be activated to synthesize DNA, RNA and chicken-specific antigens, as demonstrated by the original work of Harris and colleagues (25); thus, repressed CRBC nuclei can respond to normal regulatory signals.

We report here that subhemolytic concentrations of periodate rapidly stimulate the uptake of radioactive uridine in CRBC's and subsequently increase its incorporation into nuclear RNA. These data are the first report of early metabolic events following periodate stimulation of cells.

MATERIALS AND METHODS: Preparation of erythrocytes. Adult white Leghorn roosters or hens were bled by cardiac puncture into heparinized syringes; all further steps were done on ice. CRBC's were separated by centrifugation, the serum, buffy coat, and upper layer of red cells removed, and the cells washed once with two volumes of Eagle's MEM* with 15% heated fetal calf serum (GIBCO). After three rapid washes with PBS*, the CRBC's were used immediately.

Periodate treatment. CRBC's diluted to 10^8/ml in PBS were distributed by 5ml aliquots in screw-top tubes, and fresh aliquots of sodium periodate (Sigma) in PBS added, typically at 0.5mM final concentration. All samples were incubated for 10 min. at 23°C with frequent manual agitation and quenched by addition of 5ml 0.4% glucose in PBS. The cells were removed by centrifugation and resuspended in 10ml PBS with 15% serum before redistribution in 3ml aliquots (5×10^7 CRBC/ml). After centrifugation, the cells were gently resuspended in 3ml MEM with 15% serum, inhibitors and/or radioactive precursors as indicated.

Incubation of cells at 37° with 5% CO_2 included manual resuspension every hour. The results were not affected by incubation of the cells in suspension with continuous mixing.

Processing of labeled components. Cells were removed by centrifugation from radioactive medium and washed once with an equal volume of PBS containing 15% serum†. Acid-soluble radioactivity, RNA and DNA were determined and counted as previously described (9,22). Consecutive extractions of the acid precipitates with 95% ethanol removed much hemoglobin but no label. Radioactivity was determined in a refrigerated Beckmann liquid scintillation counter, model LS-2000, using 10ml Triton-toluene scintillation fluid.

Radioautography was performed according to Kabat and Attardi (27).

^{3}H-uridine (20-30 c/mm) was purchased from New England Nuclear Corporation, Actinomycin D from Sigma, ethidium bromide from Calbiochem, and PHA-P* from Difco.

RESULTS: Following brief periodate treatment of CRBC's, both the uptake of ^{3}H-uridine into acid-soluble pools and its incorporation into RNA are stimulated. The specific activity of incorporation is several orders of magnitude lower than observed for lymphocytes or growing cells but is reproducible for a given bird and consistent with the dormant nature of CRBC nuclei. The extent of stimulation above control values, however, is the same in this system as for lymphocytes treated with biological mitogens (23).

The dependence on periodate concentration of both rapid responses (Table I) is similar to that reported for enhancement of thymidine incorporation into lymphocyte DNA (4-6). In both systems, optimal stimulation occurs at about 0.5mM periodate, and higher concentrations are inhibitory. For CRBC's, the onset of hemolysis in PBS is one clear manifestation and explanation of the cytotoxicity observed by other workers at higher concentrations, and of the impaired incorporation of label into RNA reported here. In a comparable experiment with PHA (Table I), both uptake and incorporation are slightly affected but some hemolysis (in PBS) is evident within 10 min. of exposure to lectin.

Increased uptake of ^{3}H-uridine is typically 2- to 3-fold and manifest within 15 min., but incorporation of label into RNA is delayed about 30 min. in reaching a steady rate which is then typically 2 to 5 times control values (Fig. 1). The acid-soluble pool is completely labeled within 30 min., whereas incorporation into RNA is linear, after the initial lag, for several hours of incubation. This time course is similar to that observed for lymphocytes and biological mitogens (23).

To define more clearly the nature of labeled RNA, we have examined some effects of inhibitors (Table II). Uptake of label into cells is unaffected

†To check for hemolysis after periodate treatment, some aliquots were washed using PBS without serum. In these aliquots, but not in control samples, some hemolysis could be seen at more than 6-8 hours after treatment. No hemolysis was observed upon washing in PBS-serum.

Table II

Differential Effects of Inhibitors on Uptake and Incorporation of ^{3}H-Uridine

Experiment	Inhibitor	IO_4	Uptake[1] cpm/µg	+/-	Incorporation[2] cpm/µg	+/-
1						
CRBC	absent	-	83.3	2.0	10.8	3.5
with	absent	+	163.3		29.7	
Actino-D						
	present	-	82.1	1.9	1.7	2.2
	present	+	156.0		3.7	
2						
CRBC	absent	-	2201	1.9	497	1.8
with	absent	+	4103		873	
Actino-D						
	present	-	3092	1.9	51	1.6
	present	+	5785		82	
3						
CRBC	absent	-	1162	1.9	497	1.9
with	absent	+	2230		946	
ethidium						
bromide	present	-	934	2.2	360	2.8
	present	+	2031		1010	

A different bird was bled for each experiment, and cells were labeled for 3 hrs with ^{3}H-uridine at 5µc/ml. Drug concentrations were: Actinomycin D, 10µg/ml and ethidium bromide at 40µg/ml.

[1]Uptake represents cpm cold perchloric-acid soluble material/µg RNA. [2]Incorporation represents cpm acid-precipitable, alkali-labile material/µg RNA.

by both Actinomycin D and ethidium bromide during incubations as long as 6 hrs. However, incorporation is inhibited over 90% for all samples incubated with Actinomycin D, suggesting true incorporation into macromolecular species at DNA-dependent initiation sites. Ethidium bromide, on the other hand, may inhibit incorporation slightly (20% in controls) but has no effect on periodate treated populations, suggesting that mitochondria are not the site of induced RNA labeling (28). Radioautography confirms the biochemical data that stimulated erythrocytes incorporate uridine into nuclear, acid-precipitable material (Fig. 2 and Ms in preparation). Sucrose density gradients and polyacrylamide gel electrophoresis of extracted, labeled RNA show heterogeneous material of approximately 4s to 12s in size; after periodate, the size distribution is skewed toward larger moieties (Ms in preparation).

To evaluate the eventual fate of periodate stimulation in a cell lacking polyribosomes (24,25) and deficient in enzymes of DNA synthesis (29), we examined the assimilation of ^{3}H-thymidine; uptake is very low (about 5% the level of uridine uptake) and remains unchanged, while the incorporation of label into DNA is barely detectable and invariant for over 50 hrs. No mitotic figures accumulate in 72 hrs.

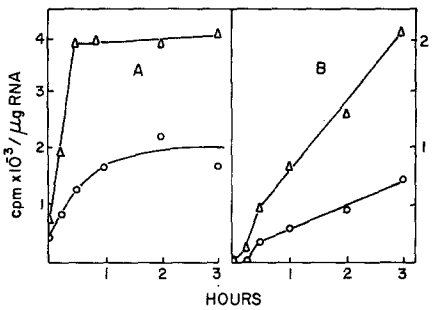

Figure 1. Assimilation of ^{3}H-uridine by avian erythrocytes: A) Uptake into acid-soluble components and B) incorporation into RNA. Cumulative time course of labeling at 5µc/ml ^{3}H-uridine at a concentration of 200 picamoles/ml. o---o control; Δ---Δ periodate-treated.

Viability of the red cell population is inaffected. For 6-8 hrs., trypan blue is excluded by over 95% of all cell populations, no hemolysis occurs in cells resuspended in PBS, and uptake of thymidine is also unaltered. We interpret these data to mean that the effects reported here for ^{3}H-uridine metabolism cannot be attributed to generalized, non-specific membrane damage. Morphological changes are limited in extent over the first few hours after treatment. We have not observed any reproducible evidence for periodate-induced cell fusion, but the treated cells are significantly more agglutinable in PBS or PBS-serum.

DISCUSSION: Chicken erythrocytes respond to mild periodate oxidation in a manner similar to that previously reported for some initial events in lymphocyte activation by lectins. Here, we report increased permeability to ^{3}H-uridine and its enhanced incorporation into RNA, both with a time course and extent of stimulation close to lectin-induced lymphocyte activation. In further analogy to lymphocyte activation, we have observed increased uptake of leucine and glucosamine into soluble pools as well as enhanced incorporation of glucosamine into macromolecules (Ms in preparation).

We believe the increased incorporation of ^{3}H-uridine into RNA reflects altered biosynthetic capacity and cannot be attributed solely to increased uptake and/or differential labeling of precursor pools by a low concentration of highly labeled uridine. To prove this point, we are presently examining the enzymatic activities of RNA polymerases in isolated nuclear preparations.

A special aspect of this system is the variability observed among data obtained from blood of different birds. Although reproducibility to a factor of 2 or 3 is obtained from sequential bleedings of one bird (compare the two sets of control values in Table I), the variation among different birds can be 20-fold for the uptake of uridine into acid-soluble material (Table II and Fig.

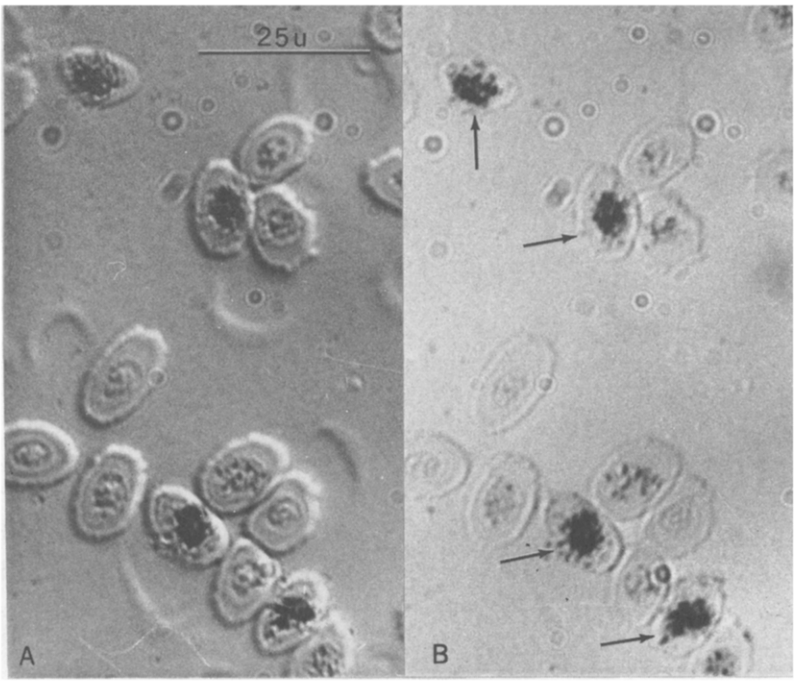

Figure 2. Radioautography of chicken erythrocytes exposed to ^{3}H-uridine after periodate treatment. 5 x 10^{7} cells/ml were exposed to 10μc/ml uridine for 3 hrs; concentrated cell suspensions were spread on coated slides, extracted with cold perchloric acid and washed with water (27) before covering with Ilford K-5 emulsion. After 14 days, slides were developed and photographed A) with Nomarski optics and B) under bright fields, magnification 1000 x.

1). As expressed by the ratio of specific activities, however, the periodate stimulation factor varies less than the absolute values obtained (Table II). Hence, the intrinsic variability among donors does not alter our conclusions.

Three criteria demonstrate that erythrocytes are the active cell type. Microscopic examination consistently shows less than 1% contamination by immature forms, lymphocytes, or other morphologically distinct cells. When lymphocyte populations are purified from the same birds and treated with periodate, the incorporation of ^{3}H-uridine is so low that only bulk contamination (40-50%) of CRBC's could account for our results (unpublished observations). Most directly, radioautography of CRBC populations has demonstrated accumulation of label in the majority of erythrocytes (Fig. 2 and Ms in preparation).

The chicken erythrocyte system offers a special opportunity to examine in isolation and in detail, the two categories of early cellular responses and thereby to study directly a central problem: is there some obligate, causal connection between the earliest surface events and rapid, subsequent changes within the nucleus, and--if so--what kinds of signals are transmitted

between these two regions of the cell. Since no protein synthesis occurs, the early responses of CRBC's occur without the diversification and amplification which lead to DNA synthesis and mitosis in other cell types. Red cells, furthermore, are choice material for the analysis of permeability or other membrane-related functions, and the dormant nucleus is capable of reactivation. Furthermore, these single cell suspensions are readily available in large quantity and quickly purified for experimental manipulation.

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