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PROTON SPIN-LATTICE RELAXATION RATES IN ERYTHROCYTES ADSORBED WITH HEMAGGLUTINATING VIRUSES

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The water proton spin-lattice relaxation rate ($1/T_1$) was measured in suspensions of erythrocytes adsorbed with the hemagglutinating Echovirus type 11. The observed $1/T_1$ enhancement, which was displaying a linear dependence on the virus concentration, was taken as a proof that the adsorption phase of the virus-cell interaction accounts, at least partially, for the previously observed phenomena in virus-infected HEp-2 cells. By injecting the Echovirus type 11 into suspensions of erythrocytes doped with Mn^{2+} or treated with concanavalin A, cytochalasin D and colchicine, it was suggested that the observed $1/T_1$ enhancement could be related to modifications of the permeability properties of the red cell membrane, as well as to movements of the cell surface receptors.

1. Introduction

The early steps of the viral infectious cycle (adsorption and penetration) correspond to complex changes in the cell membrane implying the movement of specific receptors [1].

Measurements of the water proton spin-lattice relaxation rates ($1/T_1$) in virus-infected cells were previously used to detect virus-induced structural and conformational changes of cell constituents immediately after 1 h of interaction [2–4]. The following evidence was obtained: (i) the water $1/T_1$ is made slower by the enveloped viruses and faster by the naked ones; (ii) the size of the change in $1/T_1$ is dose dependent; (iii) the change in $1/T_1$ displays the same kind of sensitiveness as the usual virological techniques; (iv) the effect may be neutralized by the virus-specific antibody.

In order to ascertain whether the adsorption process was itself sufficient to explain the observed experimental findings, we performed experiments with the model system erythrocyte-hemagglutinating virus. In fact, it is generally believed that the virus-cell interaction in this model is limited to the adsorption phase. Moreover, the interaction between the virus and the specific receptors on the red cell membrane, though quite general, may develop in different ways depending on the chemical composition of viral and cellular receptors [1,5].

For these reasons we have measured the water proton relaxation rates in erythrocyte suspensions after the adsorption with a picornavirus, the Echovirus type 11. This model system should be considered as a tool for defining the relevance of the adsorption phase in determining the virus-induced change in the cell water $1/T_1$.

2. Materials and methods

The Echovirus type 11, Gregory strain (ATCC VR-41), was propagated on 37 cells (African green

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monkey kidney) [6], grown in Eagle's minimal essential medium (MEM, Wellcome) supplemented with fetal calf serum (Labtek Eurotroph) 5% and penicillin/kanamycin mixture. Virus titers were measured in plaque forming units (PFU) on 37 cells and in hemagglutinating units (HU) towards human group O red cells (3%, v/v).

Concanavalin A, colchicine and cytochalasin D, from Sigma, were used without further purification. $Mn(ClO_4)_2 \cdot 6H_2O$, from Alpha Inorganics, was used as the Mn(II) source. All the samples were prepared in 99.75% D_2O (Merck).

Human group O erythrocytes were separated from freshly drawn blood containing 0.1 M sodium citrate, washed in isotonic phosphate buffer, centrifuged at 2000 rpm for 15 min, and suspended in deuterated phosphate buffer. Erythrocyte ghosts were prepared using the method of Dodge et al. [7].

After the adsorption with Echovirus, the cell or membrane suspension was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant was used for the virus titration in HU; the cell pellet was resuspended in deuterated phosphate buffer and used for the NMR measurements.

All the NMR measurements were performed by means of an FT-NMR Bruker WH-90 spectrometer operating at 90 MHz. The resonance frequency of the deuterium nucleus was used as 'internal lock'. The data were handled with a Nicolet BNC/12 computer having 12K memory. Spin-lattice relaxation times were measured from the partially relaxed proton spectra obtained from $(180° - \tau - 90° - t)_n$ pulse sequence. The T_1 was evaluated by a least-square analysis of the exponential curve. The error was evaluated at $\pm 4\%$ and the data are the mean value of at least three experiments.

3. Results and discussion

The water proton relaxation rate measured in whole blood and washed red cell suspensions was found to be very similar to that measured in hemoglobin solutions [8]. These findings were interpreted by suggesting that the relaxation in erythrocyte suspensions is mainly caused by water molecules interacting with hemoglobin within the cells. As a matter of fact, a strong spin transfer between protein and water protons was assumed to explain the relaxation of the hemoglobin-water proton spin system in erythrocytes [9].

When dealing with relaxation caused by equilibrium among water molecules in several environments, the model describing either the motions or the relaxation mechanisms is still a puzzling question. The equilibrium is established among at least three kinds of water molecules: extracellular, intracellular and hemoglobin-bound water. The relaxation rate is therefore determined by the relaxation in each environment, by the residence time of the water on the protein and by the residence time of the water inside the cell. The exchange rate is fast enough to give rise to a single exponential decay of the longitudinal magnetization.

We have measured the water $1/T_1$ for erythrocyte suspensions from 0.5 to 3% (v/v) in D_2O and in the corresponding ghost preparations. The results reported in fig. 1 display a linear dependence on the cell pellet dilution which is consistent with an exchange averaged relaxation of all the water

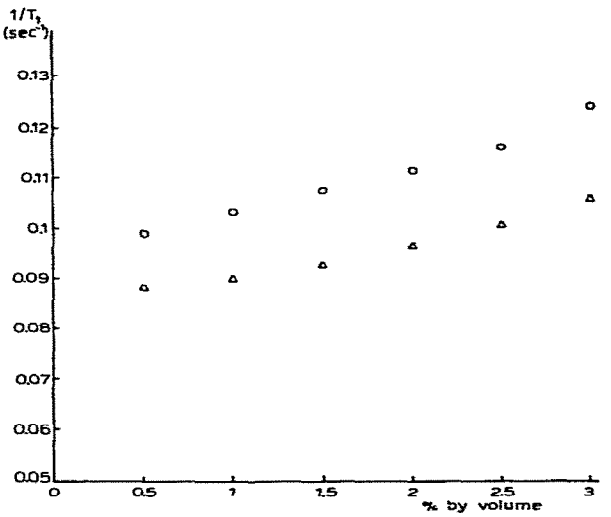

Fig. 1. Water proton spin-lattice relaxation rate ($1/T_1$) in erythrocytes (○) and erythrocyte ghost membranes (△) suspended in 99.75% D_2O at different percentages by volume. $T = 298$ K.

molecules. The $1/T_1$ values are considerably slower than those found in H_2O solution; this is to be ascribed to the isotopic dilution effect [10,11]. The water $1/T_1$ values measured in the ghost suspensions are always slower than those in red cells, confirming that the hemoglobin-bound water plays a major role in determining the proton relaxation of intracellular water.

The water $1/T_1$ values were then measured in a 3% (v/v) erythrocyte suspension and in the corresponding ghost preparation both treated for 15 min with different concentrations of Echovirus. The data are reported in fig. 2a as $1/T_{1v}$ versus HU ($1/T_{1v} = 1/T_1$ after virus adsorption—$1/T_1$ control). In both cases a dose-dependent proton relaxation rate enhancement is observed, suggesting that the virus adsorption on the cell membrane receptors is itself effective in determining a change in the water relaxation rate. The dose dependence is not linear and suggests a saturating effect due to occupation of all the cell receptors. A significant change is evident also with 0.5 HU of virus, i.e. with half of the minimal virus amount that can agglutinate the suspension of human red cells. Namely, 1 HU was corresponding to about 10^5 PFU, which is a very large amount of virions interacting with the cell membrane without producing hemagglutination. The supernatants corresponding to each NMR measurement were tested for both the hemagglutinating and infectious activities and only 1% of the virus was found at the highest concentration (8 HU).

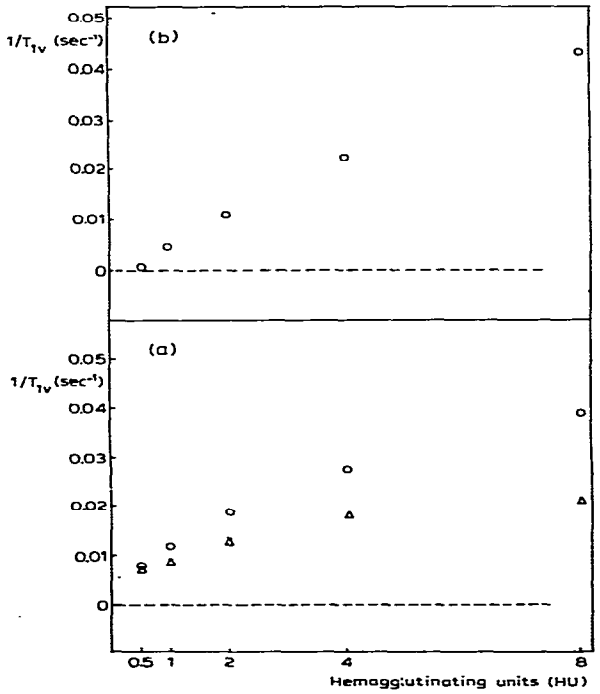

Fig. 2. (a) The water proton $1/T_{1v}$ in erythrocyte (○) and erythrocyte ghost membranes (△), both 3% (v/v) in 99.75% D_2O, treated for 15 min with different hemagglutinating units (HU) of Echovirus type 11. The dashed line gives the water $1/T_1$ in the 3% (v/v) erythrocyte control suspension. (b) The same as in (a) after elimination of viral inoculum. T=298 K.

In order to verify whether the presence of virions not bound to cell receptors could affect the observed phenomena, we measured the water $1/T_1$ in 3% (v/v) erythrocyte suspensions adsorbed for 15 min with the same amounts of Echovirus without the elimination of viral inoculum. The results are reported in fig. 2b where a change in slope and also a change in sensitivity (no effect is observed at 0.5 HU) are evident with respect to the data obtained in the previous experiment. These slight differences can be ascribed to an enhancement of the water relaxation rate caused by the unattached virions, but do not deny the statement that the virus attachment to the cell membrane brings about events reflecting a modification of the water $1/T_1$. As a consequence, the previously observed $1/T_1$ enhancement in HEp-2 cells infected with different naked viruses [2–4] arises, at least partially, from the virus adsorption to the host cell membrane.

Subsequently, we have performed experiments to distinguish between changes in the red cell water content and effects related to movements of cell receptors and/or to the cell cytoskeleton. It has long been known [12–15] that the red cell membrane is characterized by a very low permeability to the uptake of cations (negligible for many hours) and an extremely high permeability to water diffusion (the lifetime of a water molecule inside a red cell ranges from 5 to 25 ms). The addition of paramagnetic Mn^{2+} to the suspending medium has therefore been used to enhance the relaxation

rate of extracellular water allowing the evaluation of the water permeability with NMR techniques [12–15]. In fact, in the Mn-doped erythrocyte suspensions the relaxation of extracellular water is determined by the Mn^{2+} concentration, that of the intracellular one by the hemoglobin amount, and, therefore, the observed relaxation rate is unequivocally determined by the fractions of intra- and extracellular water. We have measured the water $1/T_1$ in a 4% (v/v) red cell suspension containing 8.3 mM Mn^{2+} and we have found that a single exponential could fit the observed longitudinal relaxation rate, in agreement with previous results [9]. Due to the presence of Mn^{2+} the linewidth undergoes a dramatic broadening (from 2 to 20 Hz) and the water $1/T_1$ becomes very fast (from 0.25 to 10.64 s^{-1}). The T_1 experiment is reported in fig. 3a, while a typical T_1 experiment for a 4% erythrocyte suspension containing 8.3 mM Mn^{2+} after 15 min adsorption with 8 HU of Echovirus is shown in fig. 3b. It is apparent that the relaxation rate is further quickened in the sample where Mn^{2+} was added after the virus adsorption. The data obtained with different concentrations of Echovirus are reported in fig. 4: the relaxation rate is enhanced and the line is broadened in treated erythrocytes and both $1/T_{1v}$ and $\delta\Delta\nu$ ($=\Delta\nu_{1/2}$ after virus adsorption $-\Delta\nu_{1/2}$ control) display a linear dependence on the amount of HU of Echovirus which was adsorbed on the same concentration of erythrocytes.

From these results it may be concluded that the change in the water proton spin-lattice relaxation rate observed after the virus attachment to the host cell membrane is, at least partially, arising from reduction of the water content inside the red cell. Namely, the increase in the fraction of extracellular water lets Mn^{2+} affect the relaxation of a larger number of water molecules resulting in the water relaxation rate enhancement and in a further line broadening. It is worth noting that the same findings might also be interpreted in terms of changes in membrane permeability toward the ion diffusion inside the cell, but the change in the water content seems more reasonable.

The structural and functional properties of the red cell membrane are relatively well known [16,17] and it is generally believed that the movement and

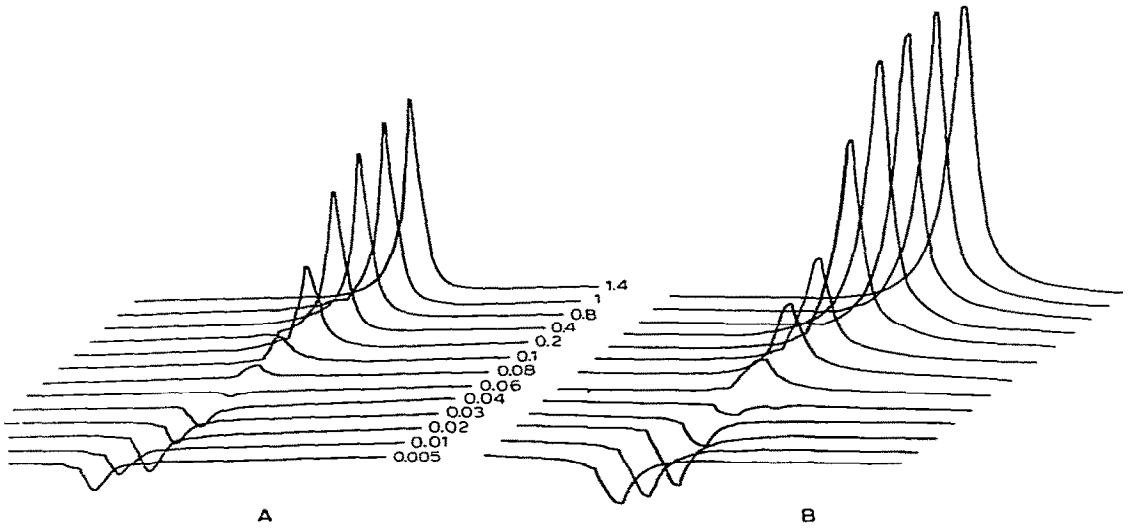

Fig. 3. (a) Partially relaxed proton NMR spectrum of water in a 4% (v/v) erythrocyte suspension in 99.75% D_2O containing Mn^{2+} in the suspending medium. (b) The same as in (a) after 15 min adsorption with 8 HU of Echovirus type 11. $[Mn^{2+}]=8.3$ mM; $T=298$ K.

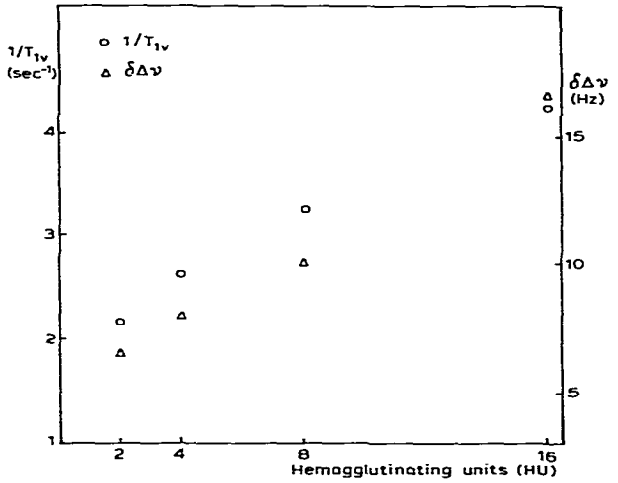

Fig. 4. The water proton $1/T_{1v}$ and $\delta\Delta\nu$ in a 4% (v/v) erythrocyte suspension in 99.75% D_2O doped with Mn^{2+}, after 15 min adsorption with different hemagglutinating units (HU) of Echovirus type 11. $[Mn^{2+}]=8.3$ mM; $T=298$ K.

the redistribution of surface receptors are driven by cytoskeleton elements [18–20]. In order to ascertain whether the virus-induced change in the water $1/T_1$ could imply a contribution from structural and conformational modifications of macromolecular cell constituents, either surface receptors or cytoskeleton proteins, we have performed experiments by utilizing the properties of concanavalin A, cytochalasin D and colchicine. In fact, concanavalin A has specific receptors on the erythrocyte membrane, causing capping and hemagglutination [21,22], colchicine depolymerizes microtubules [23] and cytochalasin D prevents the formation of actin microfilaments [24,25].

The water proton spin-lattice relaxation rate was measured in erythrocyte suspensions treated for 15 min with each of the three compounds at different concentrations. The results are summarized in table 1. Although concanavalin A is found to cause effects only at the highest concentration, it is apparent that a water $1/T_1$ enhancement is generally induced by the interaction of these substances with the red cell. Since these findings are similar to those obtained after the virus adsorption, the relationship between the two effects has been tested, as reported in table 2.

Table 1

Water proton spin-lattice relaxation rate $1/T_1$ in a 3% (v/v) human red cell suspension adsorbed with concanavalin A (Con A), cytochalasin D (Cyt D) and colchicine (Clc) for 15 min

Con A (μg/ml)	Cyt D (μg/ml)	Clc (μg/ml)	$1/T_1$ (s^{-1})
–	–	–	0.111
5	–	–	0.112
25	–	–	0.115
50	–	–	0.123
–	0.05	–	0.139
–	0.25	–	0.143
–	1.25	–	0.154
–	–	8	0.132
–	–	40	0.135
–	–	200	0.141

When the virus was adsorbed on erythrocytes previously treated with each of the three substances, the relaxation rate enhancement was much smaller than that obtained in untreated red cells. However, the change in $1/T_1$ was still greater than that observed either in the control experiment or in erythrocytes treated with each substance alone. It is likely that the three compounds affect the mechanisms through which the virus modifies the water NMR behavior, suggesting virus-induced effects on the movement of cell surface receptors as well as on the functional features of cytoskeleton elements. Since the adsorption of virus was affected only by the presence of concanavalin A, as also

Table 2

Water proton spin-lattice relaxation rate in a 4% (v/v) human red cell suspension adsorbed with 8 HU of Echovirus type 11 in the presence of concanavalin A, colchicine and cytochalasin D

	$1/T_1$ (s^{-1})	Virus adsorbed (%)
Control	0.137	–
+ Echovirus	0.196	100
+ concanavalin A [a]	0.164	75
+ colchicine [b]	0.154	100
+ cytochalasin D [c]	0.161	100

[a] 50 μg/ml.
[b] 200 μg/ml.
[c] 1.25 μg/ml.

shown in table 2, in this case only the reduced change in the water $1/T_1$ could be ascribed to a less effective adsorption.

It may be concluded that the virus attachment to the host cell membrane causes a change in the water proton relaxation rate, allowing the early detection of the infectious process. The effect (i) is due to virus-induced modification of the permeability properties of the cell membrane and (ii) needs the whole activity of microtubules and microfilaments.

Acknowledgement

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