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ON THE MECHANISMS OF ASSOCIATION OF THE MACROLIDE ANTIBIOTIC ERYTHROMYCIN WITH ISOLATED HUMAN POLYMORPHONUCLEAR LEUCOCYTES

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Abstract—In contrast to other antibiotics, the macrolide antibiotic erythromycin (ERY) has been demonstrated in previous studies to accumulate strongly in PMNs. In this study the mechanisms of association of ERY with human polymorphonuclear leucocytes (PMNs) were investigated. A kinetic approach was followed to establish the processes involved. It is argued that only passive and no active energy-dependent mechanisms contribute to the association process, since it has been demonstrated that (a) no counter-transport could be observed, (b) no consistent competition of ERY with structural analogues could be realized, and (c) no energy was required from oxidative pathways. Furthermore several other arguments point to passive mechanisms of ERY–PMN interaction. The extracellular concentration of ERY was linearly related to the degree of ERY–PMN association. The degree of association of ERY with both intact and lysed cells was dependent on its ionization state. In addition, the association and dissociation process of ERY was slow at 37°.

From these results it is deduced that the 17-fold accumulation of ERY in PMNs found at the usual *in vivo* ERY blood levels is due to binding of ERY to intracellular sites. In fact this intracellular binding might prevent intracellular activity of ERY. In addition, association of ERY with intact PMNs is inhibited by human serum, human serum albumin and α_1-acid-glycoprotein. Activation of the PMNs by phorbol ester and chemotactic peptide did not influence ERY–PMN association. Our results suggest that the intact PMN membrane permits free diffusive penetration of ERY only at physiological temperatures.

In order to obtain a more profound view on the possibilities and impossibilities of antibiotic drugs in assisting the human organism to kill bacterial pathogens, it is necessary to study the kinetics of the interaction between antibiotics and various constituents of the body. Since the peripheral blood polymorphonuclear leucocytes (PMNs)‡ form an elegant cellular defense system against microorganisms, these phagocytic cells certainly deserve attention in this respect. The PMNs combat infection and engulf and digest (cell) debris, making their way across the walls of blood vessels and migrating into the tissues to perform these tasks [1]. An important pharmacological target might therefore be the selective association of antibacterial drugs with the PMNs, since these cells can function as specific drug delivery systems to infected areas [2–8], while in other cases PMNs act as an antibiotic-proof shelter for bacteria [9, 10], depending on the accumulation level of the antibiotic within the PMNs.

The present study is centred on the association of the macrolide antibiotic erythromycin (ERY) with PMNs. The mode of accumulation of ERY base within PMNs is still incompletely understood. While PMN penetration of ERY is widely considered to occur as an active uptake process [7, 11–13], the details of this accumulation are still unclear. In particular, the role of the membrane is under question. Some investigators have argued from biochemical and electron microscope–autoradiography studies that ERY is randomly dispersed throughout the cytoplasm of the PMN [4]. Other studies report kinetic features such as egress of ERY from the PMNs and other cell types [6, 11]. The aim of the present study was to examine the influence of the intact cell membrane on the association profile of ERY and on the mechanisms of intracellular ERY accumulation. Intracellular binding of ERY to nonspecific sites appeared to determine the degree of accumulation rather than active uptake mechanisms. The cell membrane was no barrier for ERY diffusive penetration at physiological temperatures.

MATERIALS AND METHODS

Cell isolation. Human polymorphonuclear leucocytes (PMNs) were purified from heparinized human blood by a previously described technique [5] utilizing sequential dextran sedimentation, Ficoll-

‡ Abbreviations used: PMNs, polymorphonuclear leucocytes; LDH, lactate dehydrogenase; ERY, erythromycin; HSA, human serum albumin; PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; DPBS, Dulbecco's phosphate-buffered saline; DMSO, dimethylsulfoxide; C/E, cell to medium ratio; AAG, α_1-acid-glycoprotein; SD, standard deviation.

Paque (sp.g. 1.077) and Percoll (sp.g. 1.098) gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden). Final cell suspensions (20–30 × 10^6 PMNs/ml) in Dulbecco's phosphate buffered saline with 5.0 mM glucose, pH 7.4, 280–320 mOsm (DPBS), contained more than 95% granulocytes and were for at least 95% viable as judged by trypan blue exclusion and lactate dehydrogenase (LDH) activity measurement. Blood donors were healthy adult volunteers, who all gave informed consent.

Ligand-association assays. Cellular association studies of ERY were performed by the previously described silicone oil cushion centrifugation method and the double centrifugation method [5, 14]. The concentrations of ERY applied in the experiments (0.1–150 μM) were chosen on basis of known antibacterial levels *in vivo*. At time 0 an aliquot of a purified cell suspension containing 3 × 10^6 PMNs was added to the incubation medium with radiolabelled ERY. The total incubation volume was 600 μl.

For experiments with lysed cells, the PMNs were treated with rapid freezing followed by sonication. This procedure, which is not based on subcellular fractionation, completely destroyed the cells as checked microscopically [14, 15]. The formation of vesicles during lysing of the cells can not be excluded. In experiments in which assays with intact PMNs are compared with lysed PMNs, the same volume of suspension of intact PMNs and lysed PMNs was used. Measurement of cell association was accomplished by the silicone oil cushion method for intact cells and by the double centrifugation method for lysed cells [5, 14], since lysed cells cannot be centrifugated through the silicone oil. Under the experimental circumstances, the amount of sedimentable radiolabelled ligand in incubations without cells was less than 0.1% of the added amount in the incubation medium.

For the ligand-association assays in which the effect of pharmacological tools on the ERY–PMN interaction was studied, the cells as a rule were preincubated with the pharmacological probes during 5 min at 20° after which the radiolabelled ERY was added.

Intracellular volume and drug measurement. Routinely the intracellular volume of intact PMNs was measured as described before, employing [^{3}H]H_2O and hydroxy[^{14}C]methyl-inulin as markers [14, 15]. The intracellular drug concentration can be estimated by dividing the amount of cell associated drug by the intracellular volume [14]. Lysed PMNs had no intracellular volume.

LDH determination. Release of cytoplasmic LDH as a measure of integrity of the cells, was assayed by measuring the LDH catalyzed reduction of pyruvate to lactate in the presence of NADH [15].

Human serum. Human serum was prepared from the leucocyte donors as described before [14].

Composition of the incubation buffers for the pH experiments. The experiments were performed in 6 different incubation buffers with the standard composition (in mM): NaCl 150.5, KCl 2.7, $CaCl_2 \cdot 2H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.1, glucose · 1 H_2O 5.6. The desired pH was obtained by adding amounts of 66 mM solutions of $Na_2HPO_4 \cdot 2H_2O$ and KH_2PO_4 to these buffers (the osmolality of the different incubation buffers varied between 280 and 320 mOsm).

PMN stimulation. Phorbol 12-myristate 13-acetate (PMA) (1.6 mM) and 0.1 mM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) were dissolved in dimethylsulfoxide (DMSO) and stored at −70° until use. Prior to the experiments these solutions were further diluted with DPBS. In each experiment 3 × 10^6 intact PMNs were activated by contact with PMA (270 nM). FMLP, however, was added to the purified cell suspension (30 × 10^6 intact PMNs/ml) at a concentration of 0.1 μM, prior to the addition to DPBS in a total volume of 600 μl. The final concentration of DMSO (<0.02% v/v) had no detectable effect on any of the assays.

Dissociation studies. In order to investigate the reversibility of ERY cell association, the dissociation of ERY out of the cell interior and/or from binding sites was examined. After 60 min of preincubation of the intact or lysed PMNs with radiolabelled ERY at 37° ERY cell association was determined, and immediately thereafter the outwardly directed gradient of ERY was increased by 3.5-fold dilution of the cell suspension with DPBS at 37°. The dissociation of ERY at the indicated times was determined by the silicone oil cushion centrifugation method.

Competition studies. The competitive inhibition of the association of labelled ERY by unlabelled structural analogues can be used to obtain indications for the existence of selective carrier or association sites. For these experiments, ERY was added simultaneously with erythromycin propionate, erythromycin estolate, josamycin or spiramycin to the PMNs. After 5 or 90 min of incubation at 37° the degree of cell associated ERY was measured and compared with a control incubation without competitor.

Data presentation. The cell association of ERY is defined as the total amount of nmoles or pmoles associated with 3 × 10^6 intact or lysed PMNs as function of time or extracellular ERY concentration. In the experiments with intact cells the intracellular ERY concentration was defined as the total amount of cell associated ERY per intracellular volume unit. The cell to medium ratio (C/E) of ERY expresses the ratio of the calculated intracellular ERY concentration to the measured extracellular ERY concentration in the incubation medium. In order to exclude inter-experimental variations all comparisons were made within incubation sets. The results are presented as mean values ± SD. Student's *t*-test (two-tailed) was applied where indicated.

For a quantitative kinetic analysis PMNs were exposed to a wide range of ERY concentrations (0.1–150 μM) for 90 min, a sufficient incubation time for reaching a steady state. At higher ERY concentrations substantial cell lysis occurred (LDH release > 11%).

Reagents. Human serum albumin (HSA; essentially fatty acid free), α_1-acid-glycoprotein (AAG), PMA, FMLP, erythromycin base (mixture of erythromycin A, B and C), erythromycin stearate, erythromycin ethylsuccinate, 2,4-dinitrophenol, and spiramycin (mixture of spiramycin I, II and III) were obtained from Sigma (St. Louis, MO). DMSO was purchased from Merck (Darmstadt, F.R.G.). Eryth-

romycin estolate and [^{3}H]erythromycin base (sp. act. 0.28 mCi/mmol after chromatographic purification) were kind gifts of Eli Lilly and Company (Indianapolis, IN). Erythromycin propionate and josamycin base were gifts of Roussel Uclaf (Paris, France) and Heinrich Mack Nachf. (Illertissen, F.R.G.), respectively.

The small amount of ethanol (maximal final concentration of 5% v/v), in some cases employed as vehicle, did not alter cell viability. All compounds were soluble under the incubation conditions applied and they produced no alteration in pH of the medium.

The radiolabelled compounds [*N*-methyl-^{14}C] erythromycin (sp.act. 54.3 mCi/mmol) and [^{3}H]H_2O (sp. act 250 nCi/mg) were obtained from New England Nuclear (Boston, MA). Hydroxy[^{14}C]methylinulin (sp. act. 1.32 μCi/mg) was purchased from the Radiochemical Centre Amersham (Buckinghamshire, U.K.).

Radiochemical purity of [^{3}H]erythromycin and [*N*-methyl-^{14}C]erythromycin was assessed by thin-layer chromatography on Merck silica plates with the solvent system ethanol–NH_3–H_2O (90:5:5). Both compounds had >98% radiochemical purity, as determined by this method.

In order to investigate whether the degree of radiolabelled erythromycin associated with PMNs was purely erythromycin, we subjected PMN pellets after incubation to chromatographic analysis. Briefly, the PMNs were incubated with 150 μM [^{14}C] or [^{3}H] labelled erythromycin. After termination of the incubation, the cell pellet was resuspended in ethanol–NH_3–H_2O (90:5:5) by vigorous stirring. The upper layer was separated and used for chromatography, as described above. Only spots of erythromycin were detected, indicating no conversion of erythromycin during the incubation conditions.

In most experiments [^{14}C]labelled erythromycin was used. All the other chemicals were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

RESULTS

Characterization and viability of PMNs during incubation with ERY

During the incubation period of maximal 90 min at 37° with ERY concentrations of 0.1–150 μM, the viability, as measured by trypan blue exclusion, was 95% at least; immediately after the incubation the LDH release was measured which was always less than 11% of a positive control (the total LDH concentration of the same number of sonicated PMNs in DPBS) [15].

Time- and temperature-dependent association of ERY with PMNs

The experiments were carried out at an initial concentration of 25 μM ERY; this level corresponds to the concentration in human obtained after oral application of usual doses. The association of ERY with intact cells follows a saturation curve approaching equilibrium after 90 min of incubation (Fig. 1). At 37° a similar time-dependent association pattern is obtained with lysed cells. Here the initial rate of association appears to be larger than for intact cells. After 90 min of incubation the total association is about 250 pmole for both 3×10^6 intact and lysed cells. This corresponds to a C/E ratio of 17 for intact cells. The temperature dependence is substantial for lysed cells as well as for intact cells. At 24° the final association quantities are 70 pmole/3×10^6 lysed cells and 25 pmole/3×10^6 intact cells. At 4° these values are 25 and 9 pmole/3×10^6 PMNs, respectively.

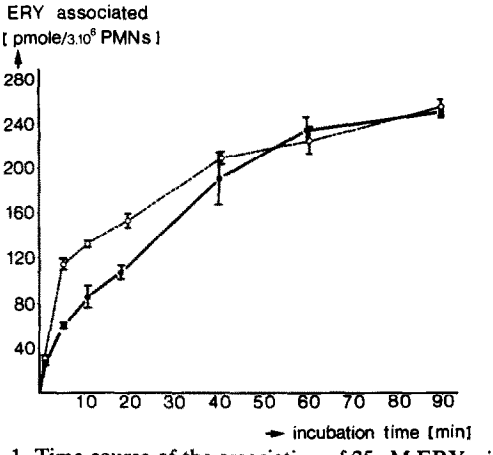

Fig. 1. Time course of the association of 25 μM ERY with 3×10^6 intact (●——●) and lysed (○——○) PMNs at 37°

These results indicate that the binding of ERY to lysed cells is temperature-dependent and suggest a major contribution of the cell membrane to the temperature sensitivity of the association of ERY with the PMN.

Concentration-dependent association of ERY with PMNs

The concentration dependence of the association has been determined in the range of 0.1–150 μM ERY. Within this range a linear relation between extracellular concentration (x μM) and associated ERY (y pmoles) after 60 min of incubation with intact cells at 37° was found that could be mathematically formulated as:

$$y = Ax + B \quad (r = 0.9999,\ P < 0.01)$$

where A is 8.0 pmole/μM and B is −1.6 pmole.

The same linearity is found at 24 and 4°, with slopes decreasing to 1.3 and 0.6 pmole/μM, respectively. At 37°, lysed cells exhibit the same concentration dependence as intact cells. Results, obtained after 90 min of incubation and expressed by the same equation yield a value of A of 11.8 pmole/μM ($r = 1.0000$) and of 12.2 pmol/μM ($r = 0.9999$) for lysed cells and intact cells, respectively. According to a Student's t-test ($P > 0.05$), there were no significant differences between both results.

Preloading of the PMNs with 100 μM [^{3}H]labelled ERY (1 hr at 37°) did not result afterwards in a substantial cell association of 1 μM [^{14}C]labelled ERY at 37° (results not shown). No uptake against a concentration gradient could be demonstrated. Neither counter-transport nor trans-stimulation could be observed.

Table 1. Time course of the effect of human serum and some serum proteins on the association of 24 μM ERY with 3×10^6 intact PMNs at 37°

Time (min)	Incubation in the presence of 97% serum	4% HSA†	0.08% AAG‡
5	252 ± 18	104 ± 54	55 ± 3
15	222 ± 89	151 ± 91	62 ± 2
60	30 ± 11	39 ± 11	48 ± 2
90	24 ± 4	19 ± 3	41 ± 2

* Results with serum or its components are expressed as the percentage of a control (i.e. without protein) association at the same incubation time (±SD).

† Human serum albumin.

‡ α_1-acid-glycoprotein.

These results suggest that no active uptake mechanisms are operating under our experimental conditions.

Influence of human serum and serum proteins on ERY association with PMNs

In order to obtain insight into the effect of components of serum on the association with intact PMNs, uptake in the presence of human serum, human serum albumin (HSA) and α_1-acid-glycoprotein (AAG) was compared with protein-free control incubations (Table 1). During the first 15 min serum and HSA appear to stimulate association. However, at longer incubation times association is decreased. AAG decreases uptake from the very beginning of the incubation. Interestingly ERY cell association at steady state was enhanced in the presence of concentrations of human serum smaller than 70% v/v.

Temperature-dependent association and dissociation studies

A further investigation of the temperature dependence of the association involved studies with lysed cells. After 20 min of incubation at 4° the association is enhanced when the incubation temperature is increased to 37°. Then in fact values are reached comparable to those obtained after permanent association at 37° (Fig. 2). The reverse experiment, lowering the incubation temperature from 37° to 4°, did not yield the reverse results: the amount of associated ERY was not significantly reduced on lowering the incubation temperature (results not shown). The dissociation from lysed cells at 37° appears to be temperature-independent. The same results were obtained with intact PMNs.

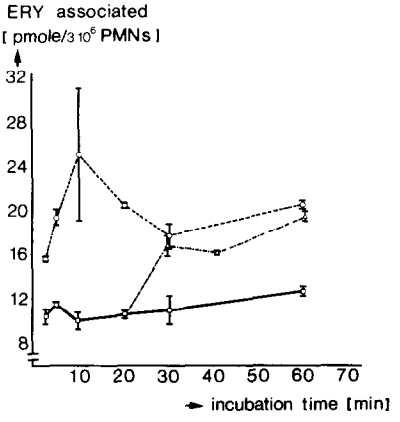

Fig. 2. Time course of the temperature-dependent association of 4 μM ERY with 3×10^6 lysed PMNs: ○——○, control incubation at 37°; □——□, control incubation at 4°; △——△, incubated for 20 min at 4° followed by an incubation at 37°.

Concentration-dependent dissociation studies

The time course of the association after 3.5-fold dilution of the incubation medium is presented in Fig. 3. While intact cells exhibit a gradual release of ERY after dilution (Fig. 3a), lysed cells behave less straightforwardly (Fig. 3b). Immediately after dilution the cell fragments release ERY, but then the association is partly re-established before dissociation continues. The net effect of the dilution is eventually the same for intact and lysed cells. However, the peculiar kinetic details immediately after dilution are only observed with lysed cells.

Influence of pH on ERY cell association

The pH of the medium was varied in order to obtain insight into the relative contributions of ionized and unionized ERY molecules to the total association. In all incubation media the viability of the PMNs after 90 min of incubation was more than 95%. As can be seen from Fig. 4, lysed PMNs show the same pH dependence of association as intact PMNs. Apart from the ionization state of the ERY molecule, in intact cells the pH gradient across the

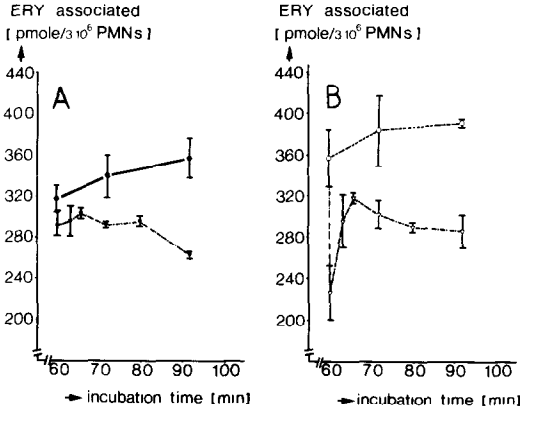

Fig. 3. Time course of the dissociation of ERY from 3×10^6 PMNs after 60 min of pre-incubation followed by a sudden 3.5-fold dilution of the medium at 37°: ●——●, control incubation of intact PMNs with 25 μM ERY; ▼——▼, incubation of intact PMNs with 25 μM ERY; at t = 60 min, the ERY concentration is decreased to 7 μM by dilution. The first data point of the dissociation curve is obtained 1 min after start of the dilution; ○——○, control incubation of lysed PMNs with 25 μM ERY; ▽——▽, incubation of lysed PMNs with 25 μM ERY; at t = 60 min, the ERY concentration is decreased to 7 μM by dilution. The first data point of the dissociation curve is obtained 1 min after start of the dilution.

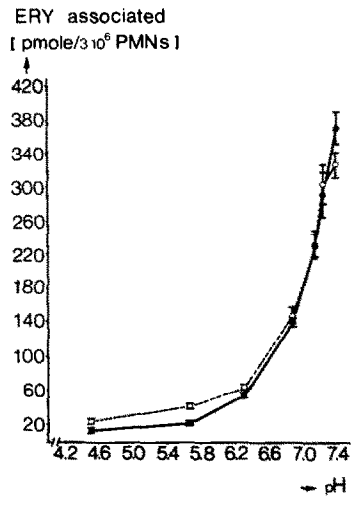

Fig. 4. Association of 25 μM ERY with 3×10^6 intact (●——●) and lysed (○——○) PMNs after 90 min of incubation at 37° as a function of the extracellular pH.

cell membrane is changed, while in lysed cells the association sites are directly influenced by pH variation. pH Experiments have also been performed at 4° (results not shown). At this lower temperature, differences between lysed and intact cells occur. While lysed cells behave similarly to the incubations at 37°, intact cells exhibit a very low degree of association all over the pH range.

Low extracellular pH, which implicates more ionized extracellular ERY, decreases the ERY association with intact cells only at 37°. The interaction between ERY and lysed PMNs is pH-dependent both at 37 and 4°, suggesting a major role for the binding of ERY as mechanism of ERY cell association.

Influence of cell function on ERY cell association

In order to investigate the influence of cell activation on the association of 25 μM ERY with intact PMNs, studies with a phorbol ester (2 nM–11 μM PMA) and chemotactic peptide (0.1 μM FMLP) were performed. These cell activators have no effect on the ERY cell association (data not shown). Likewise no significant influences were found when the cells were exposed to a wide range of 2,4-dinitrophenol concentrations (10 nM–118 μM). This compound is classically used as an uncoupler of oxidative phosphorylation and thereby as a noncompetitive inhibitor of energy-dependent transport mechanisms.

Effect of structural analogues of ERY cell association

Several structural analogues were investigated with regard to possible inhibition or stimulation of ERY cell association. The results are shown in Table 2. Some drugs tested slightly stimulate association, while others tend to inhibit. At concentration levels of 25 and 150 μM some esters of ERY such as ERY stearate and ERY ethylsuccinate, had no significant effect on radiolabelled ERY base–PMN association. The same results were achieved after 5 and 90 min of incubation.

DISCUSSION

Cellular concentrations of drugs can determine the pharmacodynamic responses. In the case of therapy for intracellular infections, antibiotics are required, which easily reach therapeutic levels within the target cell [16, 17]. Considering this fact, an attempt is made to establish the mechanism(s) of the reported accumulation of ERY in PMNs [4, 11] through a kinetic approach [14] to cell–drug interactions.

A passive process of association of ERY with intact PMNs is more plausible than an active process, since it has been demonstrated that the degree of cell association of ERY was dependent on the ionization state of ERY base (Fig. 4), which has a pK_a of 8.8 [18]. This finding which suggests diffusion of ERY into the PMN, has also been reported for ERY lactobionate [7] and clindamycin [3]. In addition, the association of ERY to and the dissociation of ERY from the intact PMN at 37° (Figs 1 and 3) was found to be slow. The similar degree of association with intact and lysed cells at steady state at 37° (Fig. 1) suggests binding of ERY to the PMN rather than active uptake mechanisms in the PMN.

Some classic experiments were performed to investigate the possible contribution of active uptake processes. The incubation temperature strongly influences the accumulation degree in the PMN. Similar findings have been reported for ERY propionate [11] and ERY lactobionate [7]. The extracellular concentration of ERY was linearly related to the degree of ERY–PMN association. This finding argues against the possibility of binding sites and active transport mechanisms over the PMN membrane saturable at the concentration range applied

Table 2. Effect of some structural analogues of ERY on the association of radiolabelled ERY (25 μM) with 3×10^6 intact PMNs at 37°. The labelled and unlabelled drugs were added simultaneously to the PMNs*

Concentration (μM)	Josamycin	ERY propionate	ERY estolate	Spiramycin I, II and III
25	1.5 ± 0.2	1.5 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
150	1.5 ± 0.2	1.3 ± 0.2	†	0.7 ± 0.1

* Results are expressed as the ratio of ERY association in the presence of the structural analogue to the association of ERY alone (±SD).

† Not determined due to cell lysis.

(up to 150 μM). In addition, neither counter-transport nor trans-stimulation could be observed, and no consistent competition of ERY with structural analogues could be realized (Table 2). Some compounds have no effect on ERY cell association, while others tend to stimulate or inhibit this process. A logical explanation for these findings cannot be given. Furthermore, we obtained evidence that ERY cell association does not require energy from oxidative pathways.

Since these results practically exclude active transport mechanisms, we have investigated the role of intracellular binding by performing experiments with lysed PMNs. Lysed cells also show a temperature-dependent association (Fig. 2). The association with lysed cells is strongly enhanced when the incubation temperature is increased from 4 to 37° (Fig. 2). When transferred from 37 to 4°, however, no reduction of association is observed which indicates that the rate constant of association is more temperature-dependent than the rate constant of dissociation. Since association is equal for intact and mechanically lysed PMNs only at 37°, in contrast to the formaldehyde-killed PMNs [11], and the association with lysed cells is higher at 24 and 4° than with intact cells, it is clear that not only the association with cell components is temperature dependent, but also the ERY penetration through the intact cell membrane. Some authors [11, 16] found a marked difference between the "cell/medium ratio" of ERY propionate of intact and lysed cells (intact > lysed). However, these authors have neglected that lysed cells have by definition no intracellular volume [14]. The only meaningful comparison in this respect is that of the amount of associated ERY with lysed and intact cells. That intracellular binding of ERY occurs is further supported by the results of the pH dependence studies. The interaction between ERY and lysed cells is pH dependent both at 37 and 4° (Fig. 4), while the pH dependence of ERY association with intact cells is only apparent at 37°. Incubation at lower temperatures decreases the diffusional flux of ERY through the intact PMN membrane as a result of a decrease of the permeability constant, in particular by the increasing viscosity of the membrane.

The gradual exchange between intracellular and extracellular ERY at 37° is evident, since it diffuses slowly out of PMNs upon lowering of the extracellular ERY concentration (Fig. 3). Contrary to these findings, experiments with lysed cells show a rapid exchange between bound and non-bound ERY (Fig. 3). The overall conclusion can be drawn that the rate of ERY penetration in and efflux of ERY out of intact PMNs is determined by a slow, non-active, passage through the cell membrane.

Physiological concentrations of human serum, HSA, and AAG strongly inhibit ERY cell association at steady state (Table 1). After oral dosing ERY is rather highly bound to plasma proteins (65–73%) [18]. AAG was reported as an important ERY binding protein in human serum [19]; during acute inflammation the serum concentration of AAG is elevated [20, 21]. Protein binding of the macrolide will not only decrease the PMN accumulation of ERY, but also the tissue penetration of the drug and its microbiological activity [22].

ERY will often be administered to patients whose PMNs are not in a resting state but have been activated by one or more pathophysiological influence(s). Experimentally PMN activation can be brought about by soluble agents such as PMA and FMLP. Both cell activators *in vitro* have no effect on ERY cell association. This observation agrees with the results of Hand and King-Thompson [12], who found no significant alteration of ERY cell accumulation after ingestion of *Staphylococcus aureus* by PMNs. After zymosan ingestion by the PMNs, a comparable slightly diminished intracellular concentration of ERY was repeated [16].

In conclusion, the cellular membrane permits free penetration of ERY only at physiological temperatures. In fact intracellular binding may prevent activity of ERY within the PMN. Binding does not occur solely to the outer surface of the cell membrane, since the temperature and pH dependent experiments point at trans-membrane diffusion. Serum proteins markedly decrease the ERY association with intact PMNs at steady state. Taken together, there is convincing evidence that ERY cell association is based on passive mechanisms, and that intracellular ERY incorporation is due to association to binding sites.

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