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The effect of receptor phase composition on the permeability of hairless mouse skin in diffusion cell experiments

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Summary

The effect of different antimicrobial agents (0.1% and 0.04% formaldehyde and 0.002% NaN_3) in the receptor phase of diffusion cells and the influence of the length of time of exposure of hairless mouse skins in the diffusion cells to the receptor phases have been evaluated by determining their effect on the flux of theophylline in diffusion cell experiments. If the length of exposure to the receptor phase was 4 h before application of theophylline, the flux of theophylline was essentially the same regardless of the antimicrobial agent used. If 0.1% formaldehyde was the agent used, the flux of theophylline remained relatively constant regardless of the time of exposure to the receptor phase. For the other two agents (0.04% formaldehyde and 0.002% NaN_3) the flux of theophylline increased sharply with increased time of exposure. This result is apparently due to microorganism growth in the receptor phases containing 0.04% formaldehyde and 0.002% NaN_3.

Introduction

Recently, a series of articles from several different research groups have appeared which have evaluated the suitability of using hairless mouse skin as a substitute for human skin in diffusion cell studies (Durrheim et al., 1980; Bond and Barry, 1988a–c; Hinz et al., 1989). A number of important questions have been addressed not only by those groups but indirectly by others as well. For example, what is the effect of prolonged hydration on the barrier function in hairless mouse skin (Behl et al., 1980; Durrheim et al., 1980; Sloan et al., 1986; Bond and Barry, 1988a; Hinz et al., 1989), and how does that effect compare with the effect in human skin (Bond and Barry, 1988a–c)? What is the effect of sequential topical treatments of the same tissue sample (Sloan et al., 1986; Bond and Barry, 1988a; Hinz et al., 1989)? What is the effect of the various vehicles that have been used on the barrier function of hairless mouse skin (Sloan et al., 1986; Sherertz et al., 1987; Bond and Barry, 1988b)? On the one hand, several groups found that the barrier function of the mouse skin significantly deteriorated with time upon hydration (Bond and Barry, 1988a–c; Hinz et al., 1989) while human skin did not (Bond and

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Barry, 1988a–c). On the other, Sloan and co-workers (Sloan et al., 1986; Sherertz et al., 1987) have reported that there was no significant difference in the delivery of theophylline from propylene glycol through hairless mouse skin regardless of whether the skins had been in contact with the aqueous receptor phase for 2, 24 or 120 h. Obviously, the different results from the various groups not only make it difficult to compare results from the various groups but it also raises the question as to whether experiments where hairless mouse skin has been used in diffusion cells for several days to study vehicle effects and repeated applications are meaningful.

After comparing the protocols used by the various groups, one major difference was apparent. They all used a different receptor phase in their studies. Thus, Bond and Barry (1988a–c) used water containing 0.002% sodium azide (NaN_3) and Sloan and co-workers (Sloan et al., 1986; Sherertz et al., 1987) used a phosphate buffer containing 0.1% aqueous formaldehyde to prevent microorganism growth, while Guy and co-workers (Hinz et al., 1989) used saline without a preservative. Other groups have used different antibacterial and/or antifungal agents to prevent microorganism growth (Mollgaard and Hoelgaard, 1983; Van der Merwe et al., 1988; Jones et al., 1989), but the protocols described by Bond and Barry, Sloan and co-workers, and Guy and co-workers are reasonably representative of the spectrum of approaches taken by workers in this area to deal with the potential problem of microorganism growth.

In order to determine the effect of the composition of the receptor phase on the permeability of hairless mouse skin, diffusion cell experiments have been run using two different concentrations of aqueous formaldehyde or 0.002% NaN_3 in a phosphate buffer receptor phase. In order to determine the effect of time on permeability, diffusion cell experiments have been run using four different lengths of time of exposure of the hairless mouse skins to the receptor phases (4, 24, 48 and 120 h) from time of killing to time of application of theophylline in a propylene glycol standard drug/vehicle combination. Since constant changing of a receptor phase that did not contain a preservative did not prevent damage to hairless mouse skin in previous experiments (Hinz et al., 1989), frequency of changing the receptor phase was not included as a viable variable in determining the cause of damage in this study. In addition, second applications of the standard drug/vehicle have been run in most instances to determine the effect of multiple applications on the effect of time and receptor phase composition on skin permeability.

Materials and Methods

Materials

The diffusion cells were Franz type cells from Crown Glass of Somerville NJ. The diffusional surface was 4.9 cm^2 and the receptor phase volume was 20 ml. A Fisher model 80 circulating water bath was used to maintain the temperature of the water-jacketed receptor phase at 32°C. Theophylline was obtained from Sigma, St. Louis, MO, and propylene glycol (PG) from Aldrich. Miscellaneous chemicals and aqueous formaldehyde were obtained from Fisher. The mice were female hairless mice (SKH-hr-1) from Temple University Skin and Cancer Hospital. Ultraviolet spectra were obtained on a Cary 210 spectrophotometer. A Zeiss Axioskop was used to examine the microorganisms using 63 × plan-apochromat and 100 × plan oil immersion objectives. Microorganisms were cultured in Difco tryptic soy broth and on Difco tryptic soy agar plates.

Diffusion cell experiments

The diffusion cell experiments were run as previously described (Sloan et al., 1986; Sherertz et al., 1987). The mice were killed by cervical dislocation and their skins were separated by blunt dissection. The skins were immediately clamped between the two halves of the diffusion cells using a rubber O-ring to seal the epidermis side to the donor half with the dermis side in contact with a receptor phase consisting of pH 7.1 phosphate buffer (0.05 M, 0.11 M ionic strength) and one of three preservatives. The three preservatives used were 0.1% formaldehyde (A, 2.7 ml of 36% aqueous formaldehyde per 1 of buffer), 0.04% for-

maldehyde (B, 1.0 ml of 36% aqueous formaldehyde per l of buffer), or 0.002% NaN_3 (C).

The skins were kept in contact with the receptor phases for 4, 24, 48, or 120 h before theophylline/PG suspensions were applied. In one set of experiments the donor sides of the membranes were also kept in contact with deionized water during the entire preapplication period (48 h). The receptor phases were changed at least three times during the preapplication period in all of the cases except where the skins were kept in contact with the receptor phase for only 4 h before the application of theophylline/PG. In that case, the receptor phases were only changed twice, but they were changed after each sample was taken during the first 12 h after the application of theophylline/PG. The receptor phases were changed periodically during the application periods in order to maintain sink conditions in the receptor phases.

After 4, 24, 48, or 120 h of contact by the mouse skins with the receptor phases, a suspension of theophylline/PG (0.5 ml) was applied to each diffusion cell. Unless otherwise specified $n = 3$. The receptor phases were sampled periodically during this 48 h first application period: 11–12 times during the 48 h, typically at 3, 6, 9, 12, 15, 21, 24, 27, 30, 36, 44 and 48 h. In all the experiments the theophylline in the receptor phase was quantitated by UV analysis at 270 nm ($\epsilon = 1.02 \times 10^4$ l/mol) as previously described (Sloan et al., 1986). In those experiments where several samples were taken without changing the receptor phase, the cumulative mg of theophylline in the receptor phase had to be calculated taking into account the samples that had been removed. This was accomplished by adding 15% (3 ml sample/20 ml receptor phase) of the mg of theophylline found in each sample taken after the receptor phase had been changed.

After the first application period the donor phases were removed with 3×10 ml of methanol. Then the receptor phases were changed. After 23 h the receptor phases were analyzed for theophylline by UV spectroscopy and the receptor phases were changed. Then, a second application of theophylline/PG was made in all the cases except for those where the first application was made after 120 h of contact with the receptor phase. The receptor phases were sampled periodically during this second application period: 5–6 times during 24 h, typically at 2, 4, 6, 12, 20 and 24 h. Theophylline was quantitated by UV analysis at 270 nm as described above.

In all cases the cumulative mg of theophylline in the receptor phases were plotted against time. Linear regression analysis of the steady-state portion(s) of each plot gave the steady-state slope in mg/h. Fluxes were obtained by dividing the slopes by the area of the diffusion cells (4.9 cm^2).

In the microbiological experiments, theophylline/PG was applied to each of the diffusion cell membranes 48 h after the mice were killed. Receptor phase samples were taken at 4, 8, 12, 20, 24, 27, 30, 33, 36, 44, and 48 h after theophylline/PG application. The samples were analyzed by UV spectroscopy and theophylline was quantitated at 270 nm as described above. The slopes and fluxes were determined as described above.

Microbiological experiments

In order to determine qualitatively if there were any differences between the effect of 0.1% formaldehyde (A), 0.04% formaldehyde (B), and 0.002% NaN_3 (C) on microorganism growth in the receptor phases, two diffusion cells using hairless mouse skin membranes were set up for each of the three different receptor phases. The handling of the mouse skins while the diffusion cells were set up for the microbiological experiments was the same as for the earlier diffusion cell experiments — no special aseptic procedure was used. In all six cases the receptor phases were sampled for the presence of microorganisms at approx. 24, 48, 72 and 96 h after the skins had been placed in contact with the receptor phases; the receptor phases were changed after each sample. The microbiological samples were taken directly from the side arm of the Franz diffusion cell using a sterile 100 µl disposable pipet tip and an Eppendorf digital pipetter whose tip had been washed with methanol and dried before each sample. The 100 µl sample was evenly streaked on a sterile agar plate with a sterile loop. Immediately after the plates were inoculated, they were sealed with Parafilm, inverted, and incubated at 37° C.

The plates were examined visually for microbial

growth every 24 h. Where possible, the plates were removed from the incubator before colonies grew to confluency so that the number of individual colonies could be estimated. No plates were removed prior to the initial 24 h period. Plates which exhibited no growth after 48 h at 37°C were considered negative. Colonies were typed based on size, color and morphological characteristics by light microscopy. Single colonies of each 'type' were selected with a sterile 1 μl loop and suspended in about 0.5 ml sterile tryptic soy broth. Sterile agar plates were streaked with these suspensions and incubated at 37°C in order to isolate pure cultures of each colony. Each isolate was then classified by gram stain (Clark, 1981), motility, and HPLC analysis of polyamine content (Busse and Auling, 1988).

In two additional experiments using only two diffusion cells each, one diffusion cell using receptor phase A and one using C were similarly monitored for microbiological growth and theophylline diffusion in each experiment.

Statistics

Statistical analysis was performed using a one-tail Student's *t*-test to compare means ± SD for flux values: values were significantly different for $p < 0.05$.

Results

Permeability

The results from the diffusion cell experiments are given in Table 1. The first application steady-

TABLE 1

Fluxes of theophylline and lag times for development of steady state

Conditions		First Application		Second Application	
		Flux (±SD) (mg/cm^2 per h)	Lag time (h)	Flux (±SD) (mg/cm^2 per h)	Lag time (h)
A [a]	4 h [b]	0.0011 ± 0.00010	5.7	0.0033 ± 0.0014	2.8
	24 h	0.0015 ± 0.00035	4.5	0.0029 ± 0.00088	2.5
	48 h	0.0017 ± 0.00022	5.2	0.0027 ± 0.00058	4.9
	48 h [c]	0.0021 ± 0.00011	8.2		
	120 h	0.0018 ± 0.00022	8.2		
	48 h [d]	0.0022 ± 0.00036	11.7	0.0039 ± 0.0011	11.2
B [e]	4 h [f]	0.0018 ± 0.00062	11.8	0.017 ±0.0080	7.6
	48 h [g]	0.034 ±0.018	15.5	0.26 ±0.12	0.8
		0.087 ±0.046	24.1		
	48 h [c,g]	0.019 ±0.0046	17.4		
		0.053 ±0.031	27.0		
	120 h [g]	0.027 ±0.0084	14.1		
		0.077 ±0.025	22.9		
C [h]	4 h [i,g]	0.0019 ± 0.00033	16.9	0.029 ±0.014	8.0
		0.0048 ± 0.0019	28.6		
	48 h	0.083 ±0.017	20.3	0.47 ±0.076	0.8
	48 h [c]	0.154 ±0.035	21.6		
	48 h [d]	0.22 ±0.031	14.2	0.23 ±0.10	7.7

[a] The receptor phase contained 0.1% formaldehyde.
[b] The length of time the hairless mouse skins were in contact with the receptor phase before the first application.
[c] Diffusion cell experiments in which receptor phase was analyzed for microbiological growth.
[d] The donor phase was in contact with deionized water for 48 h before theophylline/PG application.
[e] The receptor phase contained 0.04% formaldehyde.
[f] $n = 4$.
[g] There are three stages in the delivery of theophylline through skin. The last two stages are apparent steady-state fluxes.
[h] The receptor phase contained 0.002% NaN_3.
[i] $n = 6$.

state fluxes of theophylline from PG obtained when 0.1% formaldehyde (A) was used as a preservative in this study are essentially identical to those obtained in a previous study where 0.1% formaldehyde was also used as a preservative in the receptor phase (Sloan et al., 1986). In each of the present experiments where 0.1% formaldehyde was used, there was an initial stage and a steady-state stage for the plot of cumulative mg in the receptor phase vs time. The initial stage was curvilinear and relatively short. The steady-state stage lasted the remainder of the experiment and gave a good linear relationship between cumulative mg and time ($r = 0.999$). This result is typical of most diffusion cell experiments.

In the present study, the flux obtained from the 4 h receptor phase contact experiment was not significantly lower than that obtained for the 24 h experiment, but it was significantly lower than the fluxes from each of the remaining experiments where 0.1% formaldehyde was used as a preservative. However, there were no significant differences among the fluxes obtained from the 24 h, 48 h with or without water contact with the donor side of the membrane, 48 h with microbiological sampling, or 120 h receptor phase contact experiments.

The results from the second application of theophylline/PG to the skins that had been in contact for various lengths of time with receptor phase containing 0.1% formaldehyde were also consistent with results from the previous study (Sloan et al., 1986). There was little difference in the fluxes from the second application of theophylline/PG regardless of the time from sacrifice to first application.

The outcome was entirely different when the first application fluxes of theophylline/PG obtained using either 0.04% formaldehyde (B) or 0.002% NaN_3 (C) as the preservative in the receptor phase were considered. For instance, in experiments where (B) was used in the receptor phase, the flux of theophylline obtained from the 4 h receptor phase contact experiment was essentially the same as where (A) was used. But when the skins had been in contact with (B) for 48 or 120 h, the fluxes of theophylline from the first application experiments were significantly higher than those obtained after 4 h of contact. In addition, there were apparently three stages in the delivery profile of theophylline through the hairless mouse skin in the two former experiments. In those experiments the initial stage was followed by an apparent steady-state (second) stage that lasted through four or five sample periods which in turn was followed by another apparent steady-state (third) stage which lasted through the last three or four sample periods. The flux values obtained from these second stage steady-states using (B) and 48 or 120 h of skin contact with the receptor phases were 10–20 times greater than those obtained after only 4 h of receptor phase contact before the first application. The differences were even greater (30–50 times) if the third stage steady-state fluxes from these experiments were compared to the 4 h of contact with (B) experiment. The results from the second application experiments using hairless mouse skin which had been in contact with (B) showed that the second application fluxes of theophylline were significantly greater than the corresponding first application fluxes or second application fluxes where receptor phase (A) had been used.

Similarly, in experiments where (C) was used, three stages in the delivery of theophylline from PG were observed, but in this case after only 4 h of skin contact with the receptor phase. The values for the fluxes for the steady-state (second stage) portion of the 4 h experiments using (C) were not significantly different from the 4 h experiments where (B) was used, but the fluxes observed for the third stage were 2.7 times higher. The steady-state fluxes from the experiments where the skins were in contact with (C) for 48 h before the first application of theophylline were similar to values obtained where receptor phase (B) was used, and significantly higher than the fluxes for the delivery of theophylline in experiments using (A). The results from the second application experiments using (C) showed that the second application fluxes of theophylline were almost 10–100 times higher than the corresponding second application fluxes where receptor (A) was used.

Finally, the treatment of the donor side of hairless mouse skins with water for 48 h while the receptor side was in contact with (A) or (C) re-

sulted in a 100 times greater first application flux of theophylline through skins which had been in contact with (C) than skins which had been in contact with (A). There was no significant difference between the values for the first application fluxes and the values for second application fluxes from this experiment, and those from the similar experiment using (A) but without donor side water treatment. However, there were significant differences when comparing the similar experiments using (C): the first application fluxes were higher and the second application fluxes were lower when the donor side was treated with water.

Microbiology

The microbiological results were quite clear. In the experiment in which all three receptor phases were compared, none of the eight agar plates which had been inoculated with receptor phase samples (2 × 24, 48, 72, or 96 h) containing 0.1% formaldehyde (A) showed any bacterial growth after 48 h. At the other extreme, after only 24 h of incubation, all of the eight agar plates which had been inoculated with receptor phase samples (2 × 24, 48, 72, or 96 h) containing 0.002% NaN_3 (C) contained too many colonies to count (> 200). Receptor phase (B) exhibited an intermediate degree of bacterial growth. After 24 h of incubation, three of eight of the agar plates which had been inoculated with samples of (B) contained 100–200 colonies, and by 48 h all eight of these agar plates contained 50–200 colonies. Five morphologically distinctive colonies were isolated from samples of (B) and (C). Three types were characterized as gram negative bacilli, one as a gram negative coccobacillus and the fifth as a gram positive coccus (*Staphylococcus* sp.). We have tentatively identified the gram negative bacilli as: (a) *Serratia marcescens* — distinctive red colonies; (b) *Klebsiella* sp. — large, thick, nonmotile rods; and (c) *Pseudomonas* sp. — dark gray-yellow colonies of small, motile rods. These are consistent with bacterial flora common to the mouse, although we do not exclude other sources such as human investigators. Irrespective of the source of bacterial contamination, only receptor phase (A), containing 0.1% formaldehyde, was effective in suppressing all bacterial growth during the time frame of the diffusion experiments.

In order to ensure that the rates of delivery of theophylline through the mouse skins used in the microbiology experiments were comparable to the rates from the other diffusion cell experiments, theophylline in PG was applied to each skin used in the microbiology experiment. Those rates of delivery of theophylline were relatively close to the rates obtained after 48 h of skin contact with the receptor phase (Table 1).

Similar microbiology and diffusion results were obtained from the two additional experiments in which only two diffusion cells were run each time — one containing receptor phase (A) and one receptor phase (C).

Discussion

Although there have been some discussions about what may be causing the degeneration of hairless mouse skin in diffusion cell experiments (Bond and Barry, 1988a; Van der Merwe et al., 1988), especially compared with human skin (Bond and Barry, 1988a), no one has examined the receptor phase for microorganism growth. One group (Van der Merwe et al., 1988) has suggested that microorganism growth may be the cause, but their experiments comparing fluxes through nude mouse skin using different antimicrobial and antifungal agents were inconclusive.

The present results show that there is a significant difference between results from diffusion cell experiments in which the cell membranes were in contact with 0.1% formaldehyde and those in which they were in contact with either 0.04% formaldehyde or 0.002% NaN_3. These results suggest two possibilities. The first is that the apparent increase in permeability using either of the two latter receptor phases is due to microbe-induced damage to the dermis side of the mouse skin. This eventually affects the permeability of the stratum corneum and the epidermis with time, especially after the application of theophylline in PG to the donor side. The presence of 0.1% formaldehyde in the receptor phase is capable of preventing this damage, but neither 0.04% formaldehyde nor

0.002% NaN_3 in the receptor phase provides such protection. The second possibility is that 0.1% formaldehyde in the receptor phase is capable of chemically stabilizing the membrane by reacting with nucleophilic groups to give crosslinking of the various components of the membrane to a significantly greater extent than 0.04% formaldehyde.

However, if chemical stabilization of the membrane by 0.1% formaldehyde were the case, loss of the capability of the skin to enzymatically hydrolyze ester prodrugs might be expected. Instead, relatively complete conversion of ester prodrugs to parent drugs was observed in studies where ester prodrugs were evaluated in diffusion cell experiments for their ability to deliver the parent drug and where 0.1% formaldehyde was used in the receptor phase (Waranis and Sloan, 1987, 1988; Saab et al., 1990). In addition, in similar experiments the use of a lower percentage (0.04%) of formaldehyde led to lower conversions of ester prodrugs to parent drugs: (Sloan, K.B. and Beall, H.D., unpublished results): the opposite result expected if higher formaldehyde concentrations were chemically stabilizing the membrane by cross-linking various components of the membrane such as esterase enzymes.

The microbiological experiments suggest that the damage observed with 0.04% formaldehyde or 0.002% NaN_3 in the receptor phase is in some way due to microorganism growth. Not a single colony grew from any of the samples of the receptor phases containing 0.1% formaldehyde (A). On the other hand, there was extensive growth (too numerous to count) from samples containing 0.002% NaN_3 (C) even at the earliest sample time, while the number of colonies that grew from samples of receptor phases containing 0.04% formaldehyde (B) was intermediate. Also, it took more time for the increased permeability of the skin to manifest itself in an increase in theophylline flux in diffusion cell experiments where receptor phase (B) was used, compared to (C). This strongly suggests that the microbiological growth is the cause of the lack of membrane stability with time when 0.04% formaldehyde (B) or 0.002% NaN_3 (C) were used.

The observation of three apparent stages in the delivery profile of theophylline through hairless mouse skin when either receptor phase (B) or (C) was used is probably related to the length of time necessary for microbiological damage and the subsequent time dependent damaging effect of theophylline/PG on the integrity of the skin to occur in the presence of either receptor phase (B) or (C). For instance, the data in Table 1 for receptor phase (C) suggest that significant damage occurs within the first application period even if contact with the receptor phase is only for 4 h before application of theophylline/PG. Thus, a change in the apparent steady-state flux takes place after about 24 h to give a third stage in the delivery profile of theophylline. On the other hand, the data in Table 1 for receptor phase B suggest that no apparent damage occurs within the first application period after only 4 h of contact with the receptor phase, but does after 48 h of contact with the receptor phase.

Another possible difference between the protocols used by Guy and co-workers (Hinz et al., 1989) or Bond and Barry (1988a–c) and that used by Sloan and co-workers (Sloan et al., 1986; Sherertz et al., 1987; Waranis and Sloan, 1987; 1988; Saab et al., 1990) is that in many of the former studies the donor side as well as the receptor side of the membranes were in contact with water for variable lengths of time before application of a solute/solvent combination. The results of using either 0.1% formaldehyde (A) or 0.002% NaN_3 (C) in the receptor phase and keeping the donor side in contact with water containing no preservative for 48 h before application of theophylline/PG show that aqueous contact with the donor side of the skin does not cause damage as long as the receptor phase contains an effective antimicrobial agent. Also, pretreatment of the donor phase with water has no significant effect on the long-term stability of the hairless mouse skin.

Conclusion

Although the present experiments were limited, they clearly show the important influence of microbial growth on measured flux of compounds

through hairless mouse skin. The present experiments show that the addition of 0.1% formaldehyde to the receptor phase is effective in maintaining the integrity of the hairless mouse skin for up to 168 h of contact with an aqueous receptor phase.

It is suggested that in the future, studies in which flux through hairless mouse skin is measured should include control experiments to determine if microorganism growth in the receptor phase has indeed been controlled. The present results also suggest that studies evaluating the use of different antimicrobial agents in the receptor phase of diffusion cell experiments are warranted, and that studies to determine which microorganism found in the receptor phase is responsible for damaging the skin barrier in hairless mouse skin are also warranted.

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