Assessment of the ability of *Malassezia pachydermatis* to stimulate proliferation of canine keratinocytes in vitro

Tai-An Chen, BVM, PhD; Richard E. W. Halliwell, VetMB, PhD; Darren J. Shaw, PhD; Peter B. Hill, BVSc, PhD

Objective—To investigate the direct interaction between canine keratinocytes and live *Malassezia pachydermatis* and thereby determine the role of these organisms in the pathogenesis of epidermal hyperplasia associated with *Malassezia* dermatitis in dogs.

Sample population—Primary canine keratinocyte cultures established from skin samples obtained from clinically normal dogs.

Procedure—The proliferative response of keratinocytes co-cultured with *Malassezia* organisms for 1, 2, or 3 days was assessed by use of direct manual counting (to determine the number of keratinocytes in both the monolayer and the medium) and immunohistochemical staining techniques involving antibodies against proliferating cell nuclear antigen (PCNA) and another cellular proliferation marker, Ki-67. The potential cytotoxic effect of *Malassezia* organisms was investigated by use of an apoptosis detection kit to label keratinocytes co-cultured with *M pachydermatis* that underwent apoptosis.

Results—No stimulatory effect of *Malassezia* organisms on canine keratinocyte proliferation was detected via cell counting and immunohistochemical techniques. However, there was a significant increase in dead keratinocytes in the medium with increasing numbers of *Malassezia* organisms in the co-culture. More apoptotic cells were observed in keratinocyte monolayers co-cultured with high numbers of *M pachydermatis* than there were in monolayers cultured without *Malassezia* organisms, and the number increased after prolonged incubation.

Conclusions and Clinical Relevance—*M pachydermatis* did not stimulate canine keratinocyte proliferation in vitro. The results suggested that the epidermal hyperplasia observed in dogs with *Malassezia* dermatitis is unlikely to be caused by a direct effect of the organism on the keratinocyte cell cycle, but is likely to involve other mechanisms. *Am J Vet Res* (2004;65:787–796)

Malassezia pachydermatis is a lipophilic budding yeast that colonizes the skin and mucosal sites of healthy dogs.[1] Despite being part of the normal cutaneous microflora, it has been suggested that the yeast may become a pathogen under certain circumstances. Factors such as changes in the cutaneous microenvironment and alterations in the host defense mechanisms may trigger *M pachydermatis* overgrowth on the skin of dogs.[2,3] The term *Malassezia* dermatitis has been used to describe inflammation of the skin associated with cutaneous populations of *M pachydermatis* that are greater than those normally borne by the host; this condition has been recognized with increasing frequency, and its clinical features have been well documented.[4-6]

Techniques used to identify and quantify populations of *Malassezia* yeasts include cytologic and histologic examinations and microbial culture of specimens.[7-12] For the diagnosis of *Malassezia* dermatitis, skin biopsy techniques are generally considered to be less sensitive and reliable than other diagnostic tools because of the possible loss or disruption of the stratum corneum during processing. Nevertheless, skin biopsy samples obtained from dogs with *Malassezia* dermatitis have several characteristics. The biopsy specimens of affected skin are characterized by superficial perivascular to interstitial dermatitis with hyperkeratosis, irregular hyperplasia of the epidermis and follicular infundibula, epidermal spongiosis, and lymphocyte exocytosis.[12-16] Scott and Miller[13] described a hyperplastic dermatosis associated with secondary *M pachydermatis* infection in West Highland White Terriers. The term epidermal dysplasia was proposed to describe the epidermal hyperplasia with round-bottomed rete ridges in these dogs.

The effect of *M pachydermatis* in stimulating epidermal hyperplasia has not been extensively studied. In a preliminary report, a significant increase in a cellular proliferation marker was demonstrated in canine keratinocytes that were co-cultured with *M pachydermatis*, compared with that detected in control cells in vitro.[a] This could indicate that *Malassezia* organisms might be able to activate the proliferative cell cycle of canine keratinocytes. Recently, the epidermal dysplasia in 2 West Highland White Terriers with cytologic evidence of overgrowth of *Malassezia* organisms was reported to be reversible after antifungal treatment,[17] suggesting a possible pathogenic role of *M pachydermatis* in epidermal hyperplasia associated with *Malassezia* dermatitis.

In our experience, extracts or culture supernatants from *M pachydermatis* had no stimulatory effect on the proliferation of canine keratinocytes, regardless of whether or not they were prepared with protease inhibitors.[18] However, it was hypothesized that direct

Reveived July 17, 2003.
Accepted October 31, 2003.

From the Division of Veterinary Clinical Studies (Chen, Halliwell, Hill) and the Centre for Tropical Veterinary Medicine (Shaw), Easter Bush Veterinary Centre, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Roslin, Midlothian, EH25 9RG, UK. Dr. Chen's present address is 2F, No. 10, Lane 266, Section 4, Jen-Ai Road, Taipei, Taiwan 106.

Dr. Shaw received support from the Wellcome Trust.
The authors thank Lorna Hume, Elizabeth Thornton, and Steven Wright for technical assistance.

Address correspondence to Dr. Hill.

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contact between organisms and keratinocytes could be required to stimulate a proliferative response. The purpose of the study reported here was to investigate the direct interaction between canine keratinocytes and live *M pachydermatis* and thereby determine the role of these organisms in the pathogenesis of epidermal hyperplasia associated with *Malassezia* dermatitis in dogs.

Materials and Methods

Keratinocyte culture—Six dogs from a dog rescue center were used in this study. Specimens of healthy skin were obtained from dogs immediately after euthanasia by IV injection with 20% pentobarbital.[b] The lateral aspect of the thorax and abdomen of dogs was shaved and scrubbed with dilute chlorhexidene,[c] painted with povidone-iodine solution, and wiped with 70% ethanol. An area of full-thickness skin (approx 15 X 8 cm) was removed from each dog, dipped in PBS solution containing povidone-iodine (5:1) for 10 seconds, and washed with PBS solution containing penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL; antimicrobial combination designated as PSF[d]). The skin was then immersed in Dulbecco's modified Eagle medium[d] containing PSF.

Canine keratinocyte culture was established by use of a modification of a published protocol.[19] Briefly, the subcutaneous tissue and fat were removed by trimming, and the skin was cut into small pieces (approx 1 X 1 cm). The pieces were incubated in **William's medium E (WME)**[d] containing dispase[d] (10 mg/mL) and PSF for approximately 18 hours at 4°C. The epidermis was carefully stripped off the dermis and placed in a solution of trypsin-EDTA (0.1%/0.04%)[d] at 37°C for 10 minutes. The enzymatic effect of trypsin was stopped by addition of an excess of WME supplemented with 10% fetal calf serum,[e] 0.1nM cholera toxin,[e] 10-ng/mL murine epidermal growth factor,[d] and PSF (WME supplemented in this manner was designated complete WME). The cell suspension was filtered with a cell strainer[f] to remove hair and cell debris. The suspension was centrifuged at 700 X *g* for 10 minutes, and the cells were resuspended in complete WME. The cells were counted in a hemocytometer, and the viability was assessed by trypan blue exclusion. For primary cultures, tissue culture flasks coated with fibronectin[g] (concentration, 1 µg/cm^2) were used to enhance cell attachment to the plastic. Cells of passages 2 through 4 were utilized in our experiments.

Co-culture of keratinocytes with *M pachydermatis* for manual counting—Results of preliminary experiments indicated that a colorimetric cell proliferation assay that had been validated to enumerate canine keratinocytes in a previous study[18] was not suitable for co-culture experiments because the assay reagent reacted with the live *Malassezia* organisms (data not shown). In the present study, the keratinocytes were enumerated manually by use of a hemocytometer. Although this technique was more laborious, it allowed counting of the keratinocyte population in both the monolayer and the medium and easy discrimination between keratinocytes and yeast cells.

Keratinocytes grown in tissue culture flasks were harvested by trypsinization. Briefly, the cell monolayer was washed with sterile PBS solution (warmed to 37°C) after the culture medium was removed. An appropriate volume of trypsin-EDTA diluted in PBS solution (0.1%/0.04%) was added, and the cells were incubated at 37°C until they separated from the flask. The completion of trypsinization was determined by observation of single cells in suspension (ie, cells were not in strings or clumps) via light microscopy. Complete WME was added to the cell suspension (1:1), and the cells were pelleted by centrifugation at 700 X *g* for 10 minutes. Cells were resuspended in complete WME, counted in a hemocytometer, and plated into three 12-well plates at a density of 1.5 X 10^4 viable cells/cm^2 (1 mL/well). The plates were incubated at 37°C with 5% carbon dioxide in air. After 24 hours, the medium was replaced with complete WME without PSF. On day 3, the medium was changed again to ensure no PSF remained and the plates were incubated at 37°C while the *Malassezia* suspension was prepared.

A strain of *M pachydermatis* that had been obtained from the ear canal of a dog with *Malassezia*-associated otitis was cultured on Sabouraud dextrose agar[h] containing 20 mg/mL of chloramphenicol[i] for 48 hours at 37°C. *Malassezia* colonies were carefully harvested and suspended in PBS solution for a washing procedure that consisted of 3 cycles of centrifugation at 500 X g for 5 minutes followed by removal of the supernatant and resuspension in PBS solution. After the last washing cycle, the cells were resuspended in complete WME without PSF and counted in a hemocytometer. The suspension was adjusted to a concentration of 10^7 yeast cells/mL and serially diluted 10-fold (10^7 to 10^2). Because of the limited number of wells in a 12-well plate, 4 densities (10^7, 10^5, 10^3, and 10^2 per well [1 mL/well]) of *Malassezia* organisms were used, each of which were added in duplicate in every plate. Control samples included keratinocytes cultured in 2 mL of complete WME without PSF or yeast cells. During the next 3 days, the morphologic appearance of the culture was observed and photographed; manual counting with a hemocytometer was performed with 1 plate on each day. Before trypsinizing the cells that were attached to the plate, the medium in each well was collected and the cells in the medium were also counted. The cell viability was assessed by trypan blue exclusion. This experiment was repeated 3 times to confirm the reproducibility of results. The cells used in 3 repeated experiments were obtained from 2 different dogs and were at their second, third, and fourth passage when the experiments were conducted.

Co-culture of keratinocytes with *M pachydermatis* for immunohistochemical staining—Keratinocytes were washed, resuspended in complete WME, and counted in a hemocytometer. Cells were plated into 8-well chamber slides at a density of 2 X 10^4 viable cells/cm^2 (1.4 X 10^4 cells/well) and incubated at 37°C with 5% carbon dioxide in air. After 24 hours, the medium was replaced with complete WME without PSF and the slides were incubated for a further 3 days to allow monolayer formation. On day 5, the medium was replaced and the monolayers were co-cultured with *Malassezia* organisms at similar densities to those used in the experiments involving manual counting to determine keratinocyte numbers. A *Malassezia* suspension was prepared as described and serially diluted 10-fold to provide 2 X 10^6 to 2 X 10^2 yeast cells/100 µL; 100 µL of these diluted suspensions was added to wells containing growing keratinocytes and 300 µL of medium. Keratinocytes cultured in 400 µL of medium without *Malassezia* organisms were used as control samples. At 4, 12, 16, 24, 48, and 72 hours after co-culture, the wells were washed with PBS solution 3 times to remove nonadherent cells and the keratinocyte monolayers were fixed with 4% paraformaldehyde in PBS solution for 20 minutes at room temperature (approx 20°C). After further washing with PBS solution 3 times, the medium chambers were removed to allow the slides to be processed as standard microscope slides. Slides were stored at 4°C in 70% alcohol until used.

Immunohistochemical staining

Slides of keratinocyte monolayers were stained immunohistochemically to detect 2 cellular proliferation markers, **proliferating cell nuclear antigen (PCNA)**, and Ki-67 antigen.

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Proliferating cell nuclear antigen staining

Prior to PCNA staining, the slides underwent antigen retrieval treatment to enhance staining. Slides were rinsed with 0.05M **Tris-buffered saline (TBS)** solution (pH, 7.6) to remove any trace of alcohol and placed in a pressure cooker containing 10mM citric buffer (pH, 6.0). The pressure cooker was heated in a microwave oven at high power until the pressure valve rose, and then it was heated for a further 5 minutes at pressure. After rinsing in TBS solution, the slides were incubated with 1% hydrogen peroxide in distilled water for 5 minutes to block endogenous peroxidase activity and rinsed again. A **horseradish peroxidase (HRP)**-conjugated anti-PCNA antibody[j] was added and allowed to react for 1 hour at room temperature (approx 20°C). A negative control slide was processed by replacing the anti-PCNA antibody with an HRP-conjugated immunoglobulin negative control.[j] After another wash with TBS solution, the slides were developed with 3, 3'-diaminobenzidine[k] for 5 minutes and counterstained with Mayer's hematoxylin.[c] The number of stained cells on each slide were counted via light microscopy by a single investigator (T-AC) who was unaware of the previous treatment of each slide. Cells were regarded as PCNA-positive if the nuclei had any specific brown staining. The proliferation rate was determined by counting (at high magnification [400X]) the number of PCNA-positive cells among 100 cells in randomly chosen confluent areas. The PCNA-positive cells were also subjectively graded according to the strength of staining: cells with dark brown nuclei were classified as strongly stained cells, whereas cells with light brown nuclei were classified as weakly stained cells.

Detection of Ki-67 antigen—For Ki-67 antigen detection, slides were immersed in 10mM citric buffer in a glass beaker that was heated on a hot plate and kept boiling for 10 minutes as an antigen retrieval step. The buffer was allowed to cool for at least 10 to 20 minutes at approximately 20°C. The slides were then rinsed with PBS solution and incubated with 1% hydrogen peroxide in distilled water for 5 minutes. After rinsing with distilled water followed by PBS solution, they were blocked with horse serum[k] for 1 hour. An anti-Ki-67 monoclonal antibody[l] (diluted 1:50 with PBS solution) was used as the primary antibody, and a negative control slide was incubated with mouse primary antibody isotype control[l] for an additional 60 minutes. The reagents in the IgG kit, including a biotinylated horse anti-mouse IgG and avidin-biotinylated peroxidase complex, were used according to the manufacturer's instructions, and the duration of each incubation step was 30 minutes. The slide development and counterstaining were performed in the same manner as described for PCNA staining. One hundred cells were examined in randomly chosen confluent areas at high magnification (400X), and cells that had specific brown staining of the nuclei were regarded as Ki-67-positive. Stained cells were also classified into 2 groups (strongly or weakly stained) as described.

Apoptosis detection—The apoptotic cells in keratinocyte monolayers co-cultured with *Malassezia* organisms were detected by use of an in situ apoptosis detection kit for skin cells and tissues.[m] The staining was performed at approximately 20°C unless stated specifically, and the manufacturer's instructions were followed. Briefly, the slides were incubated with the proprietary permeabilization and blocking agent[m] for 30 minutes and then rinsed with distilled water. After incubation with 3% hydrogen peroxide in methanol for 5 minutes, slides were washed with PBS solution and incubated with **terminal deoxynucleotidyl transferase enzyme (TdT)** labeling buffer for 5 minutes. The labeling reaction mix containing brominated deoxynucleoside triphosphate mix, TdT enzyme, and TdT labeling buffer was added and allowed to react for 45 minutes at 37°C. A positive control was generated by adding nuclease into the labeling reaction mix, and a negative control was generated by omitting the TdT enzyme. The labeling reaction was stopped by adding TdT stop buffer, and after 5 minutes, the slides were washed with PBS solution. Slides were then incubated with biotinylated mouse monoclonal anti-bromodeoxyuridine antibody for 45 minutes at 37°C and washed with PBS solution. Bound bromodeoxyuridine was detected after incubation of slides with HRP-conjugated streptavidin for 10 minutes and washing with PBS solution followed by distilled water. The color was developed with TACS blue label[m] for 5 minutes, and the slides were rinsed with distilled water before counterstaining in eosin-based Red Counterstain C[m] for 5 minutes. When identifying apoptotic cells, the whole area of a well on the chamber slide was scanned via light microscopy at 100X magnification. Cells that were condensed and had increased Red Counterstain C uptake, compared with most cells, or had blue nuclear staining were regarded as apoptotic.

Statistical analyses—To investigate the significance of the relationship between the number of keratinocytes per well and the density of *Malassezia* organisms and the relationship between the number of keratinocytes per well and the duration of *Malassezia*-keratinocyte co-cultures, standard generalized linear models were used. The numbers of keratinocytes were log-transformed to normalize the data, and computer software[n] was used to perform the analyses. The potential effect of 3 repeated experiments was taken into account by including replicates in the model as a factor at the beginning of the analyses. Two factors, logarithm of *Malassezia* densities and day of co-culture, were then added into the model sequentially. The suitability of the model was confirmed by considering the error structures, which were found to be normal, and the *P* values associated with fit of the model were determined via analysis of variance F test. To investigate the changes in the proportion of cells that were weakly or strongly stained for PCNA or Ki-67 as a function of time and *Malassezia* concentration, generalized linear models with binomial errors (positive or negative) were used, with the *P* values associated with fit of the model determined by use of the χ^2 test. To investigate changes in the number of apoptotic cells as a function of time and *Malassezia* concentration, generalized linear models with Poisson errors were used, with the *P* values associated with fit of the model determined by use of an AVOVA F test. In all analyses, values of $P < 0.05$ were considered to be significant.

Results

Effects of *M pachydermatis* on cultured canine keratinocytes—The 3 experiments to evaluate the number of viable keratinocytes in monolayers co-cultured with or without *Malassezia* organisms for 1, 2, or 3 days revealed similar results **(Fig 1)**. From day 1 to day 3, there was a significant ($P < 0.001$) increase in the number of keratinocytes in the monolayers. However, at each time point, there was a significant ($P < 0.001$) decrease in the number of keratinocytes in the monolayers as the density of *Malassezia* organisms was increased. Analysis of the number of keratinocytes in the culture medium (cells separated from the monolayer) revealed similar results in the 3 separate experiments. In contrast to findings among the cells in the monolayer, a significant ($P < 0.001$) increase in the number of dead keratinocytes was detected in the medium as the density of *Malassezia* organisms was

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increased. Nevertheless, there was no significant (P = 0.103) change in the number of viable keratinocytes in the medium as the number of *Malassezia* organisms increased. Overall, there was a significant increase in the number of dead ($P < 0.001$) and viable (P = 0.012) keratinocytes in the medium during the 3 days of culture.

Keratinocytes cultured with 10^5 or 10^7 *Malassezia* organisms showed minimal growth during the 3-day culture, compared with control cells and those cultured with lower densities of the organism (Fig 1). Microscopic examination of the monolayers revealed that the higher densities of *Malassezia* organisms not only inhibited cell growth, but also were associated with morphologic abnormalities in the keratinocytes **(Fig 2)**.

Detection of proliferation markers in keratinocytes co-cultured with *M pachydermatis*—To investigate the proliferative activity of keratinocytes co-cultured with *Malassezia* organisms within 24 hours of co-culture (which might not be reflected by an increase in cell numbers), immunohistochemical techniques involving antibodies against cell proliferation markers were used to assess the proliferation rate of keratinocytes. In the slides stained with anti-PCNA antibody, PCNA-stained cells had brown-staining nuclei, whereas PCNA-negative cells appeared blue (the color of the counterstain; **Fig 3**). All cells stained blue in the negative control slide. Between 80% and 90 % of the keratinocytes were PCNA-positive (Fig 3), regardless of the density of *Malassezia* organisms used and at all time points **(Fig 4)**. There was no significant ($P < 0.421$) difference in the number of strongly or weakly stained cells with time; however, despite the apparent similarity in values among concentrations of *Malassezia* organisms, there was a significant ($P < 0.021$) decrease in the number of strongly or weakly stained cells as *Malassezia* concentration was increased.

Similarly, Ki-67-positive cells stained brown, compared with unstained cells and cells in the negative control slide, which were both blue **(Fig 5)**. Results of staining of keratinocytes with anti-Ki-67 antibody were similar to results obtained with anti-PCNA antibody staining. Approximately 80% to 90 % of the cells in the monolayers co-cultured with or without various densities of *Malassezia* organisms at 4, 12, 16, and 24 hours after co-culture were Ki-67-positive **(Fig 5 and 6)**. As with PCNA staining, there was no significant ($P < 0.124$) change in the number of weakly or strongly Ki-67-stained cells with time, but there was a significant ($P < 0.006$) negative relationship between the proportion of stained cells and *Malassezia* concentration. Keratinocytes cultured with 2×10^6 *Malassezia* organisms showed a slightly lower proliferation rate after 12 hours, and fewer strongly stained cells seemed to be among these keratinocytes, compared with the other concentration groups.

Detection of cell death in keratinocytes co-cultured with *M pachydermatis*—High numbers of dead cells were detected in the media associated with keratinocyte monolayers that were co-cultured with high numbers of *Malassezia* organisms. To further investigate this phenomenon, an apoptosis detection kit was used to label dying keratinocytes in co-culture. Stained cells had blue staining of the nuclei (indicative of fragmented nuclear chromatin, which is characteristic of apoptosis) detected by the labeling process mediated by TdT enzyme **(Fig 7)**. The positive control slide was treated with nuclease, and all of those cells had blue nuclear staining. No blue-stained cells were

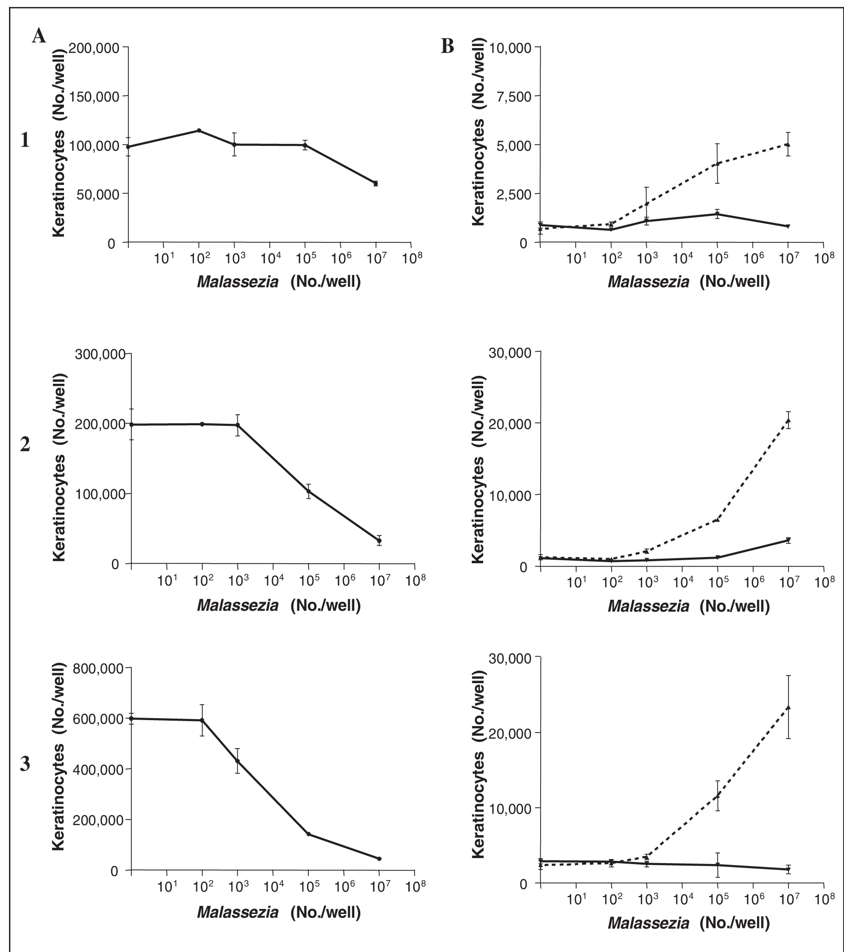

Figure 1—Representative data obtained from 3 repeated experiments illustrating the number of viable keratinocytes in the cell monolayer (A) and the number of dead and viable cells in the culture medium (B) after co-culture with 0, 10^2, 10^3, 10^5, or 10^7 *Malassezia pachydermatis* for 1 (upper panels), 2 (middle panels), and 3 days (lower panels). In section B, dead cells are represented by the dotted line and viable cells are represented by the solid line. Each datum point represents the mean and standard error of the mean of duplicates.

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present in the negative control slide, which was generated by omitting TdT enzyme in the staining procedure.

The effect of *Malassezia* organisms on canine keratinocytes was investigated at 4, 12, 16, 24, 48, and 72 hours after co-culture (**Fig 8**). The number of apoptotic cells significantly ($P < 0.034$) increased with time and with *Malassezia* concentration. Furthermore, the interaction between time and concentration was also significant ($P = 0.014$). Within 24 hours of culture, more apoptotic cells were detected in keratinocyte monolayers co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms, compared with control slides or cells co-cultured with lower densities of *Malassezia* organisms (Fig 8). The number of apoptotic cells in monolayers co-cultured with 2×10^2 to 2×10^4 *Malassezia* organisms also gradually increased during this period. At 48 hours after co-culture, the monolayer co-cultured with 2×10^3 *Malassezia* organisms had the highest number of apoptotic cells (compared with the other *Malassezia* culture concentrations), and the number of these cells continued to increase between 48

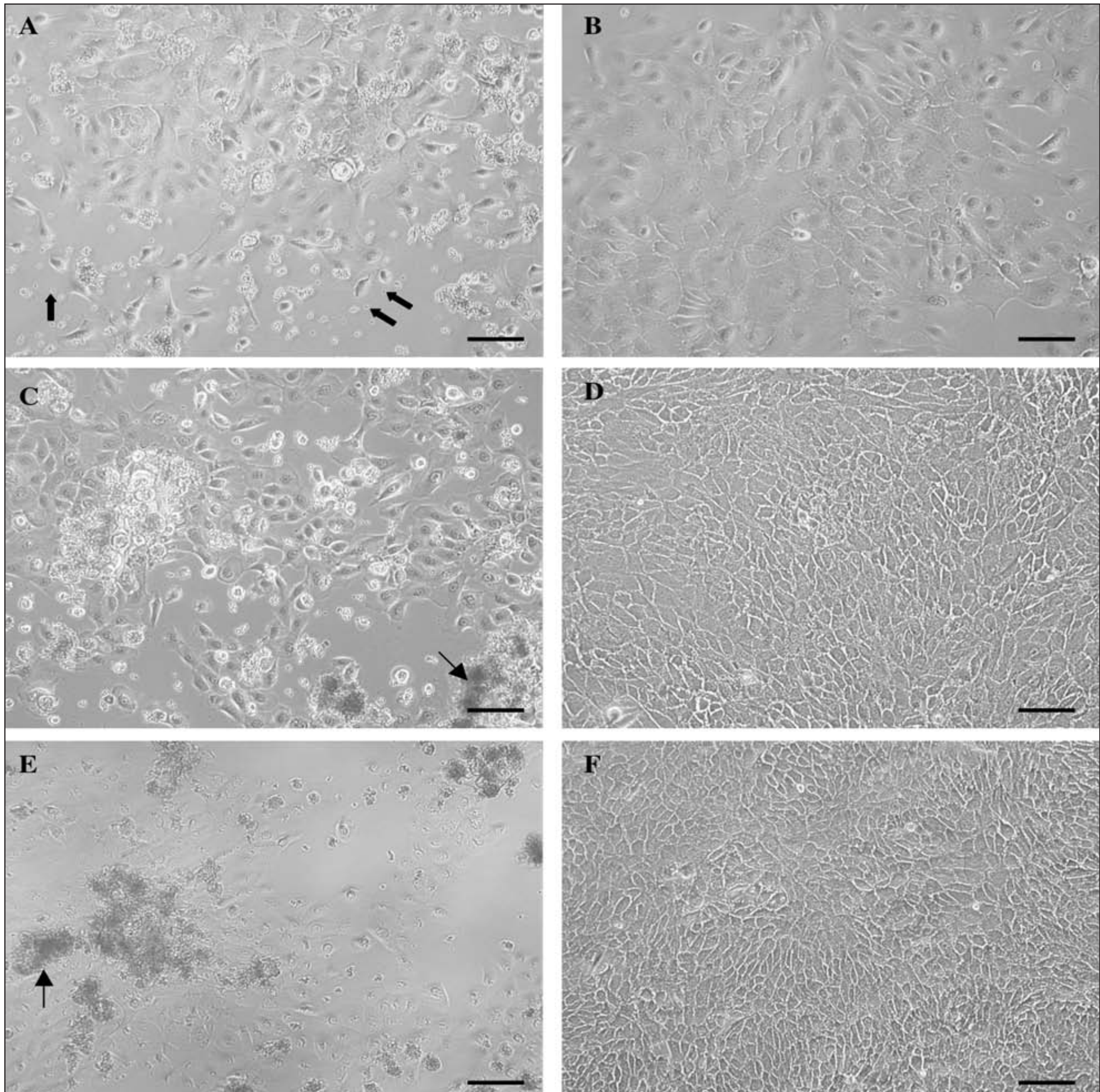

Figure 2—Photomicrographs of cell cultures of canine keratinocytes co-cultured with or without *M pachydermatis*. A—Keratinocytes co-cultured with 10^7 *Malassezia* organisms for 1 day. Notice the discrete *Malassezia* organisms (arrows). B—Keratinocytes cultured without *Malassezia* organisms for 1 day (control). C—Keratinocytes co-cultured with 10^7 *Malassezia* organisms for 2 days. Notice some clusters of *Malassezia* organisms (arrow) are present. D—Keratinocytes cultured without *Malassezia* organisms for 2 days (control). Notice that the culture is nearly confluent. E—Keratinocytes co-cultured with 10^7 *Malassezia* organisms for 3 days. Notice that cell density does not appear to be increased, compared with initial findings after 1 day of co-culture, and the keratinocyte culture is morphologically abnormal. Clusters of *Malassezia* organisms are evident (arrow). F— Keratinocytes cultured without *Malassezia* organisms for 3 days (control). This culture has dense cell populations; some cells are smaller than others. In all panels, bar = 100 µm.

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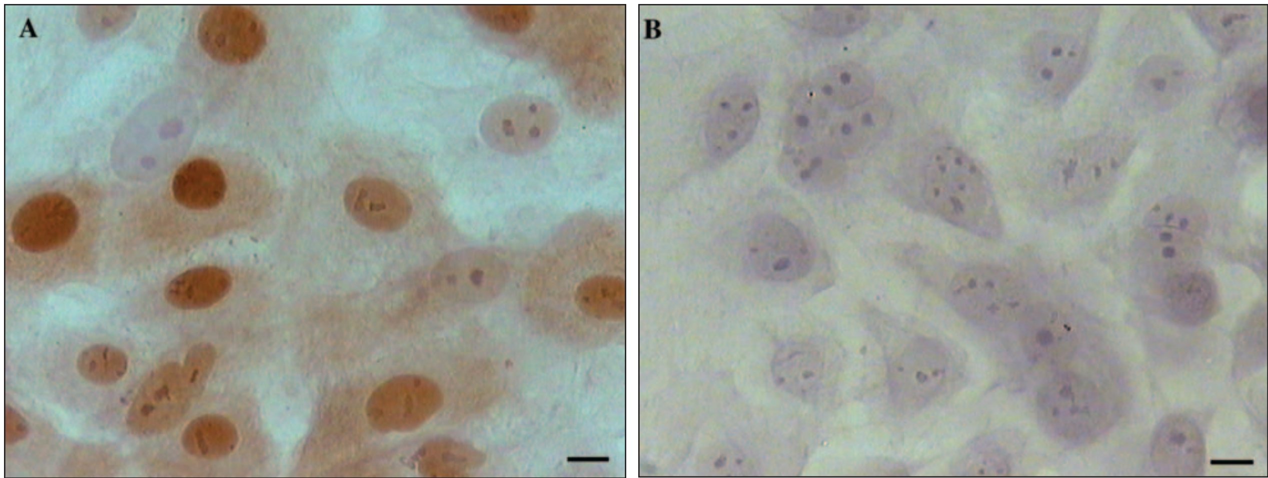

Figure 3—Photomicrographs of canine keratinocytes co-cultured with *M pachydermatis* and subsequently stained immunohistochemically to detect proliferating cell nuclear antigen (PCNA). A—Results of staining with anti-PCNA antibody of a keratinocyte monolayer co-cultured with 2 X 10^3 *Malassezia* organisms for 16 hours. Notice that 80% to 90% of the keratinocytes are stained brown. Unstained cells appear blue, the color of the counterstain. B—Keratinocytes stained with the antibody-negative control. Notice that all cells are unstained. In both panels, bar = 10 µm.

and 72 hours after co-culture. In contrast, lower numbers of apoptotic cells were detected in keratinocytes co-cultured with 2 X 10^5 or 2 X 10^6 *Malassezia* organisms at 48 and 72 hours after co-culture. However, the total number of cells attached to the slides was much lower in these 2 groups, presumably a result of the inhibitory effect of high densities of *Malassezia* on keratinocyte growth.

Discussion

In the study of this report, viable *M pachydermatis* organisms in co-culture with canine keratinocytes did not result in enhanced keratinocyte proliferation in vitro. This finding was similar to that of our previous study,[18] in which neither extracts nor culture supernatants of *M pachydermatis* had a stimulatory effect on the proliferation of canine keratinocytes. In the study of this report, results of the manual counting experiments and the immunohistochemical analyses of proliferation markers PCNA and Ki-67 indicated a significant decrease in the number of proliferating keratinocytes when they were co-cultured with high numbers of *Malassezia* organisms. These results were not in agreement with those of a preliminary study[a] that indicated a significant increase in expression of Ki-67 in canine keratinocytes that were co-cultured with *M pachydermatis*, compared with Ki-67 expression in keratinocytes cultured alone in vitro (although the same antibody was used in both studies). As the details of the preliminary study have not been reported in full, it is difficult to provide possible explanations for this discrepancy in results.

The results of the study of this report may indicate that *M pachydermatis* has no direct effect on keratinocyte proliferation either in vitro or in vivo. If this is so, the epidermal hyperplasia observed in *Malassezia*

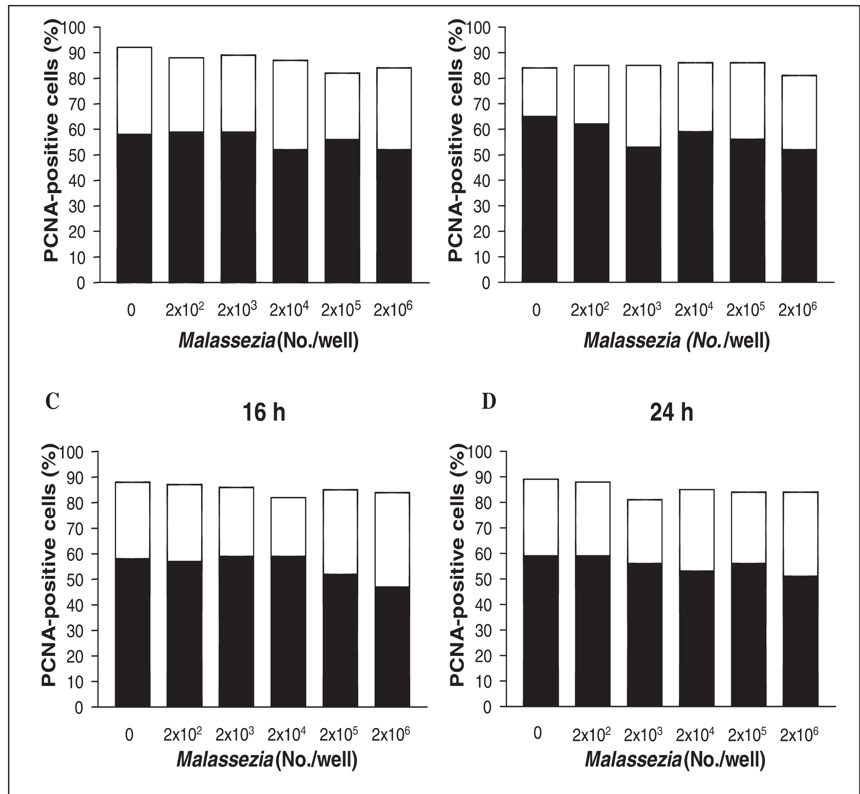

Figure 4—Percentage of PCNA-positive cells detected among canine keratinocytes co-cultured with or without *M pachydermatis* for 4 (A), 12 (B), 16 (C), and 24 (D) hours. Notice that approximately 80% to 90% of the cells in keratinocyte monolayers that had been co-cultured with or without *Malassezia* organisms were PCNA-positive. However, the number of strongly (black bars) and weakly (open bars) stained keratinocytes decreased significantly as the density of *Malassezia* organisms used in co-culture increased.

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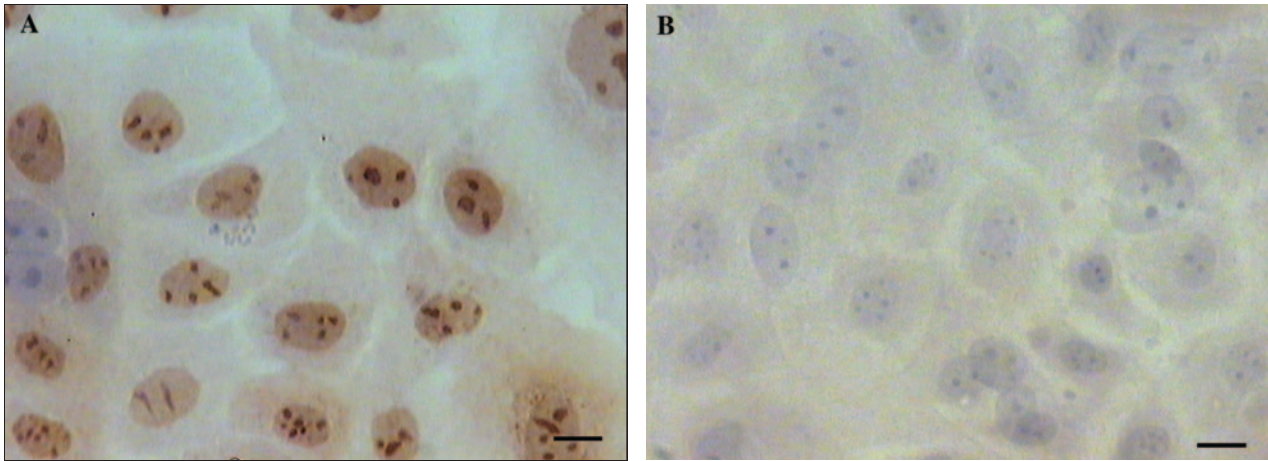

Figure 5—Photomicrographs of canine keratinocytes co-cultured with *M pachydermatis* and subsequently stained immunohistochemically to detect a cellular proliferation marker Ki-67. A—Results of staining with anti-Ki-67 antibody of a keratinocyte monolayer co-cultured with 2 X 10^3 *Malassezia* organisms for 16 hours. Notice that 80% to 90% of the keratinocytes are stained. B—Keratinocytes stained with the antibody-negative control. Notice that all cells are unstained. In both panels, bar = 10 µm.

A 4 h
Ki-67-positive cells (%)
0 10 20 30 40 50 60 70 80 90 100
0 2x10^2 2x10^3 2x10^4 2x10^5 2x10^6
Malassezia (No./well)

B 12 h
Ki-67-positive cells (%)
0 10 20 30 40 50 60 70 80 90 100
0 2x10^2 2x10^3 2x10^4 2x10^5 2x10^6
Malassezia (No/well)

C 16 h
Ki-67-positive cells (%)
0 10 20 30 40 50 60 70 80 90 100
0 2x10^2 2x10^3 2x10^4 2x10^5 2x10^6
Malassezia (No./well)

D 24 h
Ki-67-positive cells (%)
0 10 20 30 40 50 60 70 80 90 100
0 2x10^2 2x10^3 2x10^4 2x10^5 2x10^6
Malassezia (No/well)

Figure 6—Percentage of Ki-67-positive cells (black bars represent strongly stained cells; white bars represent weakly stained cells) detected among canine keratinocytes co-cultured with or without *M pachydermatis* for 4 (A), 12 (B), 16 (C), and 24 (D) hours. Notice that approximately 80% to 90% of the cells in keratinocyte monolayers that had been co-cultured with or without *Malassezia* organisms were Ki-67-positive. Nevertheless, canine keratinocytes co-cultured with high densities of yeast cells had significantly fewer stained cells.

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dermatitis in dogs is perhaps likely to be a result of the inflammatory response stimulated by the organism itself or the underlying diseases in affected animals. Alternatively, *M pachydermatis* may have no direct effect on keratinocytes in vitro, but could have a direct effect in a living epidermis that has a blood supply and an associated functional immune system. This possibility is supported by results of in vivo studies[20,21] in experimental animals in which infection with *Candida albicans* was associated with increased epidermal proliferative responses, compared with that detected in unaffected animals; furthermore, the epidermal proliferation appeared to be enhanced by the host's immunologic response to experimentally induced cutaneous candidiasis.[20,22] It is also possible that the expression of virulence factors by *M pachydermatis* could be different in tissue culture medium, compared with the virulence factors expressed in its normal environment of a living epidermis, which contains wax, sebum, sweat, and sloughing skin cells. To investigate these possibilities, further in vitro and in vivo studies would be required.

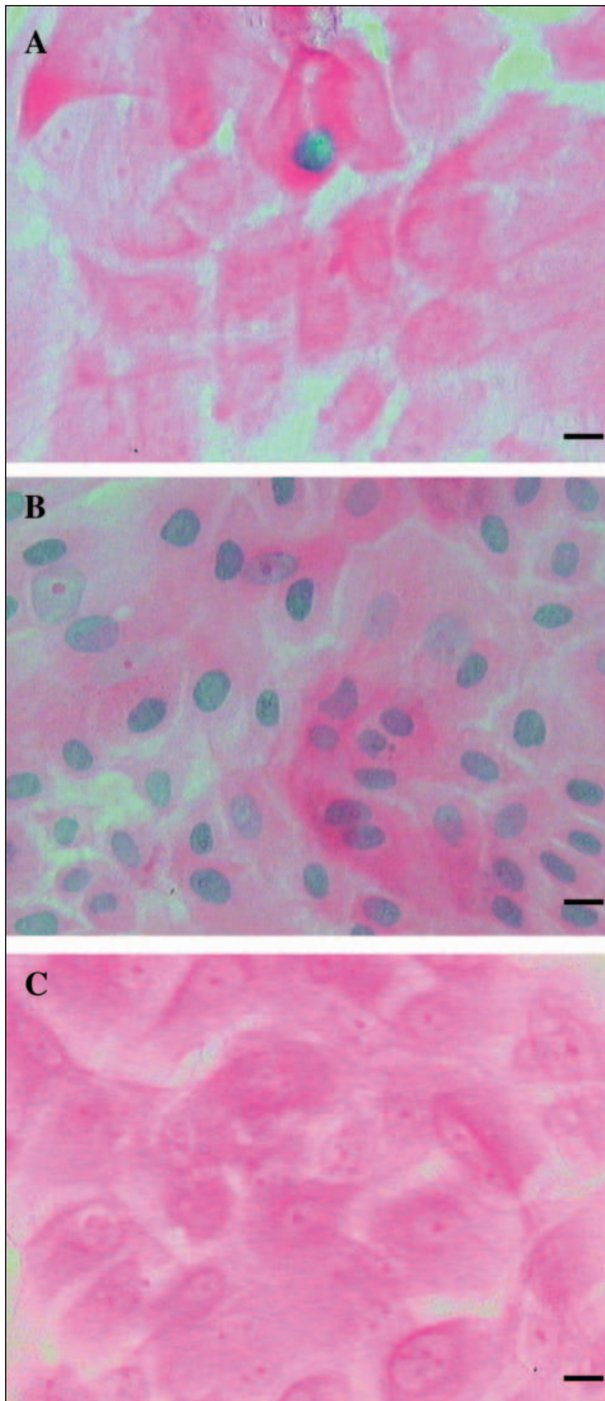

Figure 7—Photomicrographs of canine keratinocytes co-cultured with *M pachydermatis* and subsequently assessed for apoptosis by use of an in situ apoptosis detection kit for skin cells and tissues. A—After co-culture, notice that apoptotic cells have blue nuclear staining and pink cytoplasm. B—Positive control slide. Notice all cells have blue stain in their nuclei (a result of the use of nuclease). C—Negative control slide. Notice that no blue staining is detectable in the nuclei and all cells are stained pink (the color of the counterstain). In all panels, bar = 10 µm.

The dose-dependent increase in dead keratinocytes observed in the culture medium indicated that co-culture with *M pachydermatis* at high densities caused keratinocytes to undergo cell death and detach from the monolayer. Results of immunohistochemical staining also indicated that more apoptotic cells were present in the keratinocyte monolayers co-cultured with high numbers of *Malassezia* organisms than in the keratinocyte monolayers cultured without fungal organisms; after prolonged incubation, the number of apoptotic cells in the keratinocyte monolayers co-cultured with high numbers of *Malassezia* organisms increased. Although decreased numbers of apoptotic keratinocytes were observed in the specimens that were co-cultured with 2×10^4, 2×10^5, or 2×10^6 *Malassezia* organisms at 48 and 72 hours, compared with the numbers detected at a density of 2×10^3 (which might be explained by a decrease in the number of attached cells), our data indicated that as the density of *Malassezia* organisms used in culture increased, the viability of the keratinocytes decreased. Various mechanisms might explain why keratinocytes underwent apoptosis and detached from the culture plates. First, it is important to note that the in vitro experimental system used in the study of this report is a restrictive and artificial environment with finite amounts of nutrients. Competition for nutrients between *Malassezia* organisms and keratinocytes, accumulation of cellular waste products, and pH changes in the medium could all have influenced the viability of the keratinocytes. Undoubtedly, further studies involving heat-killed or nonpathogenic organisms, nutrient-depleted medium, or pH controls would be required to investigate these possibilities. Alternatively, *Malassezia* organisms might have specific effects on keratinocyte viability or adhesion. For example, *Malassezia* organisms might affect the function of transmembrane adhesion proteins of keratinocytes. Adhesion to the substratum is required for keratinocyte growth, and loss of attachment would cause a rapid growth arrest[23]; therefore, the increasing number of dead cells in the medium associated with high concentrations of *Malassezia* organisms could be explained by impairment of adhesion to the culture plate. However, the identity of such an adhesion molecule is not known and remains to be elucidated. Another explanation could be that *Malassezia* organ-

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isms had a direct cytotoxic effect on the keratinocytes that resulted in apoptosis and shedding of cells into the medium. In a study[24] to investigate the effect of *M furfur* on a human keratinocyte cell line, it was determined that high ratios of those yeast cells to human keratinocytes in culture were associated with a necrotic effect on the keratinocytes. Finally, *M pachydermatis* could be internalized by canine keratinocytes, as occurs with *M furfur*,[24] and induce keratinocyte apoptosis and result in cell detachment.[24] Necrosis and apoptosis have also been associated with invasion of human keratinocytes by Staphylococcus aureus.[25,26] The potential biological significance of these effects remains open to question because apoptosis is not a

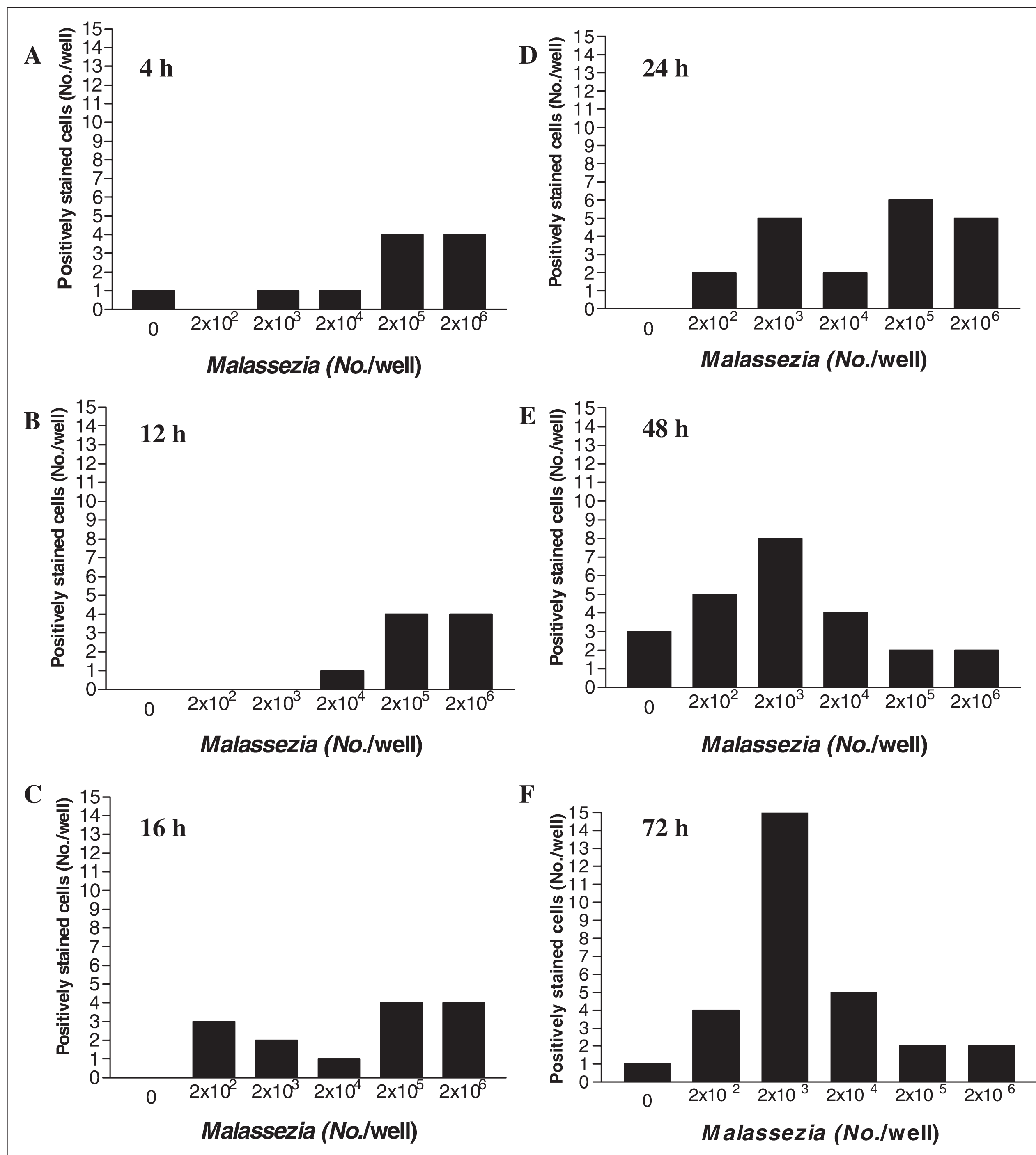

Figure 8—The number of apoptotic cells detected among canine keratinocytes co-cultured with or without *M pachydermatis* for 4 (A), 12 (B), 16 (C), 24 (D), 48 (E), and 72 (F) hours. Within 24 hours of co-culture, the highest numbers of apoptotic cells were detected in the keratinocyte monolayers co-cultured with 2×10^5 or 2×10^6 *Malassezia* organisms. At 48 and 72 hours after co-culture, the highest number of apoptotic cells was detected in the monolayers co-cultured with 2×10^3 *Malassezia* organisms, whereas the monolayers co-cultured with 2×10^5 or 2×10^6 yeast cells had low numbers of apoptotic cells, probably because of the low total number of cells in these co-cultures.

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characteristic feature detected histologically in sections from the skin of dogs with *Malassezia* dermatitis. Further histologic examination of specimens prepared with May-Grumwald Giemsa stain or sections examined via electron microscopy to demonstrate the internalization of *M pachydermatis* by canine keratinocytes, as has been performed with *M furfur*,[24,27] might provide additional evidence for this phenomenon.

Overall, our data indicated that *M pachydermatis* does not accelerate the proliferation of canine keratinocytes in vitro. Additional data regarding the role of *M pachydermatis* in epidermal hyperplasia associated with *Malassezia* dermatitis might be obtained from investigations that involve living epidermis (skin explants) or an air-medium interface culture system. The role of *M pachydermatis* as a pathogenic factor in the pathogenesis of *Malassezia* dermatitis in dogs may be further elucidated via investigation of the effect of the organism on cytokine production and expression of adhesion molecules by canine keratinocytes.

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[a]von Tscharner C, Wyder M, Busato A, et al. Proliferation characteristics of canine keratinocyte cultures infected with *Malassezia pachydermatis*, in *Proceedings*. 15th Annu Am Acad Vet Dermatol Am Coll Vet Dermatol Meet 1999;107–108.

[b]Euthatal, Merial, Harlow, Essex, UK.

[c]Hibiscrub, ZENECA Ltd, Macclesfield, Cheshire, UK.

[d]PSF, Dulbecco's modified Eagle Medium, Williams Medium E, Trypsin/EDTA, Murine epidermal growth factor, Life Technologies, Paisley, Glasgow, UK.

[e]Fetal calf serum, Cholera toxin, Mayer's hematoxylin, Sigma, Poole, Dorset, UK.

[f]70-µm cell strainer, Becton-Dickinson, Oxford, Oxfordshire, UK.

[g]Courtesy of Dr. R. C. McKenzie, Department of dermatology College of Medicine and Veterinary Medicine, University of Edinburgh, Roslin Midlothian, UK.

[h]Sabouraud dextrose agar, Oxoid, Basingstoke, Hampshire, UK.

[i]Intramycetin, Parke-Davis Veterinary, Pontypool, Gwent, UK.

[j]HRP-conjugated anti-PCNA, Dako, Ely, Cambridgeshire, UK.

[k]DAB, Vector Elite ABC mouse IgG kit, Vector Laboratories Ltd, Peterborough, Cambridgeshire, UK.

[l]Anti-Ki-67 monoclonal antibody, Zymed Laboratories Inc, South San Francisco, Calif.

[m]DermaTacs, Cytonin, R & D Systems Europe, Abingdon, Oxfordshire, UK.

[n]S-plus 2000, Insightful Corp, Seattle, Wash.

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