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PRINCIPAL INVESTIGATOR: IOANNIS V. YANNAS

PI ADDRESS: Massachusetts Institute of Technology
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Cambridge, Massachusetts 02139

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A method for separating the Langerhans cell population from a mixed dermal and epidermal cell population was studied. The long term objective of this research is the design of skin grafts which are donor-independent. This research is based on the hypothesis that the Langerhans cells in the skin are the most significant contributors to the phenomenon of skin graft rejections. Several methods were considered before designing a monolayer panning technique. The panning technique, based on the highly specific affinity of the monoclonal antibody OKT-6, proved to be very effective in achieving a cell separation. The results indicate that a single exposure to the antibody-covered surface is able to remove 72% ± 7% of the Langerhans cells from the dermal population. A second panning was demonstrated to remove 60% ± 11% of the remaining Langerhans cells. A third panning removed 66% ± 16% of the remaining Langerhans cells. The panning technique was able to reduce the average Langerhans cell content from 7.4 ± 0.8% to 0.28 ± 0.1% after successive pannings. The panning technique did not allow for the recovery of viable Langerhans cells once they had been positively selected.

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1. INTRODUCTION

A patient whose skin has been damaged from burns or abrasions must be promptly treated to reduce the risk of shock, massive fluid loss, and widespread infection. The use of xenografts has been attempted and is still being used today, but they provide a temporary solution. Xenografts, usually porcine skin, can provide a temporary functional cover, but must soon be removed as the patient's immune system rejects the graft [1,2].

Two approaches have been taken to overcome this hurdle. First, suppression of the patient's immune system has allowed for a longer graft residence by delaying the host's immune rejection of the donor graft. The objection to this approach is that it leaves the already endangered patient even more susceptible to other infections. [1,2].

The second approach is to make the grafts less antigenic to the patient and eliminate any type of immune response against the transplanted skin. This has led to the development of the autograft. The autografting procedure entails removal of a partial-thickness piece of skin from the patient followed by grafting onto the wounded area. The objections to this procedure are that it subjects an already traumatized patient to a serious operation and, as in the case of massively burned patients, donor sites from which to harvest skin may not be available. [1,2].

In response to this need for functional, non-antigenic skin replacement, a graft which can delay wound contraction and scar formation has been developed. The graft is a polymerized network of cross-linked Type I collagen and chondroitin 6-sulfate (a glycosaminoglycan, GAG) which has the optimal mean pore size, and degradation rate to provide a biologically active graft [3-5]. This Stage I matrix was recently subjected to a randomized clinical

trial to evaluate its use in the treatment of massively burned patients. The trial included 106 patients who were treated in 11 clinical centers. Heimbach *et al.* [6] reported the results of this trial and concluded that

> "in patients with major burns, the collagen-GAG copolymer matrix permitted early wound closure with as good a take as allograft, and when covered with a subsequent epidermal graft it provided a permanent cover that was at least as satisfactory as currently available skin grafting techniques and used donor grafts that were thinner and left donor sites that healed faster."

Previously, Burke *et al.* [7] had reached a similar conclusion at the completion of a 10-patient trial. One disadvantage of Stage I matrices relative to autografts was that "for patients with moderate burns who could not be covered in one sitting by conventional methods the second operation (covering with an epidermal graft) delayed definitive coverage and could lengthen hospital stay" [6].

The second generation of collagen-GAG matrices (sometimes referred to as Stage II artificial skin) overcame this objection to Stage I matrices. Stage II matrices are Stage I matrices which have been seeded with autologous (same animal or same patient) dermal and epidermal cells [9]. Seeding is accomplished by a centrifugation procedure which deposits the cells in an optimal location within the porous collagen-GAG layer [9]. Stage II matrices are capable of arresting completely contraction of full-thickness skin wounds in guinea pigs and inducing regeneration of a dermis and an epidermis [9]. The regeneration of skin is not complete: hair follicles and skin adenexa are not formed. However, ultrastructural studies have shown that the new skin is a very close replica of intact skin and is distinctly different from scar [10]. Stage II matrices act as if they were a model extracellular matrix (ECM), perhaps a crude basement membrane, which induces skin regeneration in

an animal model where such an event is well known not to occur spontaneously [10].

A disadvantage in the clinical use of Stage II matrices is that the seeding of cells requires a relatively elaborate 60-minute laboratory manipulation of a biopsy from the patient (to separate out and inoculate the cells into the matrix) before the graft can be used to cover the wound. In its contemplated use in field hospitals it would be much more convenient to make use of grafts which have been stored as seeded matrices (conceivably in a refrigerated state), ready for almost immediate use without the inconvenient requirement for laboratory facilities. The attempt to overcome this disadvantage has led to the effort to develop donor-independent Stage III matrices, i.e., collagen-GAG matrices which have been seeded with heterologous cells at the time of fabrication, have been stored at -80°C, and can be used on demand without adding to the complexity of field treatment.

To prepare a donor independent Stage III matrix it is necessary to solve the problem of rejection of heterologous cells which must be seeded into the matrix. The detailed role of the immunocompetent Langerhans cells in the phenomenon of skin graft rejection is not completely defined (see below). However, we are testing the hypothesis that Langerhans cells make all or most of the clinically significant contribution to rejection of heterologous skin grafts; and that separation of these cells from the population which is seeded into the collagen-GAG matrix can produce a graft which performs as if it were a Stage II graft (i.e., seeded with autologous cells).

Clearly, therefore, the objective of this research was to obtain a population of dermal and epidermal cells which is free of Langerhans cells, seed this modified population of cells into a collagen-GAG matrix and study

the ability of these matrices (seeded with heterologous cells) to induce skin regeneration, using as controls matrices seeded with autologous cells.

2. REVIEW OF THE METHODOLOGY OF SEPARATION OF LANGERHANS CELLS

Because Langerhans cells have been difficult to isolate, research into their origin and function has been rather slow. Following Birbeck's development of a means of positive identification of these cells, the study of Langerhans cells has increased [11]. Only recently has an immunological role for the Langerhans cells been suggested and investigated by researchers such as Billingham and Silver [12]. Silberberg and others have demonstrated the Langerhans cell's ability to phagocytose, concentrate, process and present antigen to the T-cells in lymph nodes, suggesting a strong role for the Langerhans cell as a specialized macrophage which can evoke a cell mediated immune response [13,14].

The role of Langerhans cells in graft rejection has been suggested by studies which have demonstrated that macrophage and dendritic cells expressing the Ia (HLA-DR in man) antigenic markers are responsible for stimulation of cell mediated immunity [15,16]. The use of Ia antisera and complement has been reported to permit transplantation across major histocompatibility barriers without immunosuppression of the recipient [17]. Further evidence is supplied by the fact that Ia antisera has been demonstrated to enhance skin, as well as kidney grafts [18,19,20].

It seems reasonable to conclude that removal of the Ia bearing cells in the graft prior to transplantation would serve to reduce antigenicity and perhaps allow transplantation across major histocompatibility lines. Hammerling has shown that Ia expressing cells are present in normal epidermis

[21]. Many researchers have independently demonstrated that Langerhans cells and their precursors express the Ia markers exclusively in the dermal population [22,23,24]. This suggests that removal of the Langerhans cells from the population before grafting would eliminate Ia bearing cells. It is hypothesized that a graft seeded with such a population might not evoke, or might reduce the degree of, a cell-mediated immune response in heterografts.

The task of removing a subset of a population based on different antigenic markers immediately suggests the use of monoclonal antibodies to differentiate between cell types. The use of an anti Ia nonoclonal antibody would seem to be the most straight forward approach to separating out the population of Langerhans cells. However, a recent report indicates that T6 (CD6 in humans) is a superior Langerhans cell-specific marker which can be used to achieve identification and separation [25].

The separation can be achieved in a number of ways. The most promising and practical means of sorting cells is to take advantage of their surface markers rather than their ultratructual or histochemical properties. Flow cytometry using both forward and orthogonal light scattering properties has been used to separate the epidermal population into smaller subsets [26]. An improvement on the flow cytometry technique, the fluorescence activated cell sorter (FACS) has been successfully employed by others to yield greatly enriched populations of Ia^+ and Ia^- epidermal cells [27,28]. The FACS relies upon fluorescein isothiocyanate conjugated (FITC) monoclonal antibodies which bind to the specific markers such as the Ia or T6 determinant.

We have utilized a means of separation very similar to that used by Scheynius *et al.*, referred to as the monolayer panning technique [29]. Briefly, the principle behind this technique is to attach the monoclonal antibody specific for the cell marker to a solid surface such as a polystyrene

petri dish or flask. After the antibodies have adhered, the mixed cell suspension is exposed to the surface, and the antibodies are allowed to bind. The adherent cells are expected to be positively selected for by the monoclonal antibodies and the supernatant cells are expected to be free of the selected cells providing the correct conditions are met. A more detailed treatment of the technique can be found elsewhere [30].

3. MATERIALS AND METHOD

a. Cell Donors. Harvesting of skin was performed according to an established protocol [9]. White, female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were maintained in accordance with the regulations of the Massachusetts Institute of Technology's Division of Comparative Medicine and the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Prior to harvesting of cells, the animals were shaved and depilated using a commercially available hair removal product (Nair, Carter Products, Irvine, CA). The animals were anesthetized with a mixture of nitrous oxide and methoxy fluorane and then briefly scrubbed with betadine solution. A split-thickness graft was obtained using a Ghoullian knife with a 0.8 cm shim. The dermatomed skin (approximately 2 cm^2) was stored in sterile phosphate buffered saline (PBS) (Gibco, Grand Island, New York) on ice.

b. Cell Preparation. Basal cells were isolated from the skin biopsy following modified procedures from the literature [31]. The skin biopsy obtained from the donor was covered with approximately 235 mls of 2.5% trypsin (Gibco, Grand Island, New York) and incubated for 40 minutes at 37°C . Following incubation, the epidermal layer was easily removed and discarded

using sterile forceps. The dermal tissue was transferred to a sterile capped tube containing approximately 25 mls of Dulbeco's Minimal Essential Medium (DMEM) (Gibco, Grand Island, New York) containing penicillin and streptomycin and supplemented to 10% (volume) with fetal calf serum (Hazleton Research Products, Denver, PA). The tissue preparation was agitated to dislodge cells for one minute (Vortex Genie, Scientific Industries, Bohemia, NY). The suspension was then filtered through a single layer of cheesecloth to remove any residual dermal tissue. The insoluble residues were discarded, and the remaining cell suspension was retained for further treatment. This cell suspension comprised about 70% basal cells [10,31]. Less than 10% of these basal cells are expected to be Langerhans cells or their precursors.

c. Preparation of monoclonal antibodies and the monolayer surface [30]. The anti T6 monoclonal antibody, OKT-6 (Ortho Diagnostic Systems, Raritan, New Jersey) was prepared according to the suppliers' instructions and then transferred into a 25 cm^2 T-flask (Corning Glass Works, Corning, New York) and stored at 4°C until needed. It was anticipated that the antibodies would be adsorbed onto the polystryrene surface. When the surface was prepared for use, the liquid was poured off into a fresh T-flask which was stored until needed. The flask to be used was rinsed three times with PBS before each use.

d. Panning [30]. The cell suspension was counted and adjusted to a cell density not exceeding 1.5×10^7 cells per ml. A volume of 3 ml was transferred into the coated T flask and allowed to incubate at 4°C for 15 minutes. The suspension was gently swirled and then allowed to incubate for another 15 minutes. Following this incubation period, the supernatant was sampled and retained for further analysis. The panning procedure was repeated twice and samples were obtained as before.

e. Staining. The technique for staining the cell suspensions is derived from the literature [32]. Samples of the cell suspension were stained both before being exposed to the panning surface and after each incubation. The stain used was an FITC conjugated anti-T6 monoclonal antibody, OKT-6 (Ortho Diagnostic Systems, Raritan, New Jersey). The FITC OKT-6 was prepared according to the supplier's instructions and stored at 4°C. The samples of the cell suspension were centrifuged for 10 minutes at 4°C, 800g. The supernatant was decanted, and the pellet stained with 50 µl of OKT-6 diluted 1:100 with PBS. After incubating for 30 minutes at 4°C, the samples were centrifuged as before, and the pellets rinsed twice in PBS. Following the final rinse, the samples were resuspended in 50 µl of nonfluorescing mounting medium (Aquamount, Lerner Laboratories, New Haven, CT).

f. Counting Samples. Stained samples were scored on an immunofluorescent microscope with a high pressure mercury lamp an 500nm (Carl Zeiss Instruments, New York, NY). Quantitative results were obtained using a hemocytometer (Reichert Scientific Instruments, Buffalo, New York) and photographs were taken on a Nikon 35 mm camera using P800/1600 color reversal film (Eastman Kodak, Rochester, New York). The data presented below are based on results obtained after screening approximately 18,000 cells totally under both white light and fluorescent excitations. At low magnifications, the positive staining cells' fluoresecence signals were too weak to register. Cells were viewed and photographed at 400X.

4. RESULTS

The results of the experiments indicated that the original mixed population contained 7.4 ± 0.8% (n=5,628) Langerhans cells. Following a

single panning, the Langerhans cell content of the population was reduced to 2.0 ± 0.3% (n=5,137) Langerhans cells, and a third panning reduced it further to 0.28 ± 0.1% (n=3,067). These results are presented graphically in Figure 1 and Figure 2 which chart the average Langerhans cell content of the population after successive pannings.

The removal of Langerhans cells from the dermal population is evidenced by photographs both before and after panning. The photographic prints were not available at the time of preparation of this report and will be presented later. The photographs indicate that the monolayer panning method results in reduction of the number of positively staining cells and produces a field lacking fluorescence.

In Experiment 1, the original population which contained 8.63% (n=1,089) Langerhans cells was exposed to the panning surface to yield a population containing 1.62% (n=1,539) Langerhans cells. Figure 3 shows the percent of the total population determined to be Langerhans cells for Experiment 1. Figure 4 shows the percentage of Langerhans cells removed by the single panning exposure for Experiment 1.

Experiment 2 was carried out with consecutive exposures to the same panning surface in order to determine the efficacy of successive pannings. In Experiment 2, a sample initially containing 7.38% (n=2,033) Langerhans cells was reduced to 2.46% (n=1,585) Langerhans cells after one panning. A second panning reduced this number further to 0.74% (n=1,492) and a third panning yielded a population with 0.37% (n=1,361) Langerhans cells. Figure 5 shows the percent of the total population determined to be Langerhans cells after each successive panning. Figure 6 shows the percentage of Langerhans cells removed by each panning for Experiment 2.

Similar results were obtained for Experiment 3. A sample starting with 6.26% (n=2,506) Langerhans cells was reduced to 2.04% (n=2,013) Langerhans cells after one exposure to the panning surface. The next panning reduced this number to 1.05% (n=1,903), and finally to 0.18% (n=1,706) Langerhans cells after the third panning. Figure 7 shows the percent of the total cell population determined to be Langerhans cells after each of the three successive pannings. Figure 8 shows the percentage of Langerhans cells removed by each panning in Experiment 3.

The performance of the panning surface can be quantified in a similar manner. The panning surface is able to remove 72 ± 7% of the Langerhans cells with one exposure. The monolayer surface is able to remove 60 ± 11% on the second exposure, and 66 ± 17% on the third exposure.

5. DISCUSSION.

The monolayer panning technique used in this experiment is an efficient means for selecting the Langerhans cell subset from a population of basal epithelial cells. The mean Langerhans cell content of the panned samples was significantly lower than the mean Langerhans cells content of the non-panned samples ($p<<0.0001$, $r=0.8945$, $n=10$). The advantage of this method over other techniques such as flow cytometry or affinity chromatography is that this separation procedure does not significantly affect the viability of the cell sample. In addition, the procedure can be carried out under sterile conditions. The limitation with this technique is that the selected cells are not viable after they are recovered. Analysis of the selected cells revealed that almost all cells were lysed and fragmented. The fraction of lysate which

was recovered did stain strongly, but no intact cells were found for further study and identification.

The cells which have been selected by the panning technique are expected to be Langerhans cells based on previous studies which report that in the dermis, these cells are the exclusive expressors of the T6 antigenic marker which is serving for the basis of the selection procedure [33,34,35]. The use of T6 as a means of identification of Langerhans cells has been established by Harrist *et al.* [36] who demonstrated that the anti-T6 monoclonal antibody reacts with all epidermal Langerhans cells in normal skin. In his experiment, selection of the Langerhans cells using an anti-T6 monoclonal antibody was followed by immunoelectron microscopy to demonstrate that the cells selected by the antibody did indeed have the ultrastructural identifiers previously used to identify Langerhans cells. Prior to the development of monoclonal antibodies the Langerhans cells were not identified via their cell surface markers; but rather via their electron microscopic features [11,37] which include: 1) the presence of a small distinctive organelle, the Langerhans cell (Birbeck) granule, 2) a clear cytoplasm, a lobulated nucleus, and 4) the absence of desmosomes, tonofilaments, premelanosomes or melanosomes [38].

6. CONCLUSIONS

a. A method for separating Langerhans cells from a population of dermal/epidermal cells has been developed. This method will be used in future studies to prepare collagen-GAG matrices which are seeded with cell suspensions enriched in basal and almost completely free of Langerhans cells. Suchs tudies are expected to lead to development of a new skin graft which can

induce skin regeneration with a single application in patients with massive skin loss in field hospitals.

b. The separation method exposes the cell population to a solid surface on which the monoclonal antibody OKT-6 has been adsorbed (panning). The results showed that in a population containing about 7% Langerhans cells (LC) which was treated sequentially by the panning procedure, the LC content was reduced to about 2.5% (first cycle), 0.7% (second cycle) and 0.4% (third cycle).

7. RECOMMENDATIONS

Since the motivation for this effort has been to produce a model matrix which can be transplanted across major histocapatibility lines, the next step will be seeding of the purified population into collagen-GAG matrices, to prepare heterografts which can be tested *in vivo*.

If the grafting experiments show differences between autologous and heterologous grafts, other methods, discussed earlier will be combined with the monolayer technique in an effort to yield improved results. The passive method of monolayer panning employed in this report may be easily supplemented with the use of Ia antisera. The use of antibody dependent, complement mediated, cell lysis may allow for a very sharp decrease in Langerhans cell numbers by actively eliminating Langerhans cells from the population. The same monoclonal antibodies will be used for an experiment of this sort. Perhaps the monolayer panning technique in conjunction with complement mediated cell lysis (or perhaps complement lysis alone) would allow an even more efficient means for obtaining a population free of Langerhans cells.

Such a population would lead to a more definitive test of our original hypothesis concerning the preparation of donor-independent skin grafts.

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FIGURE 1

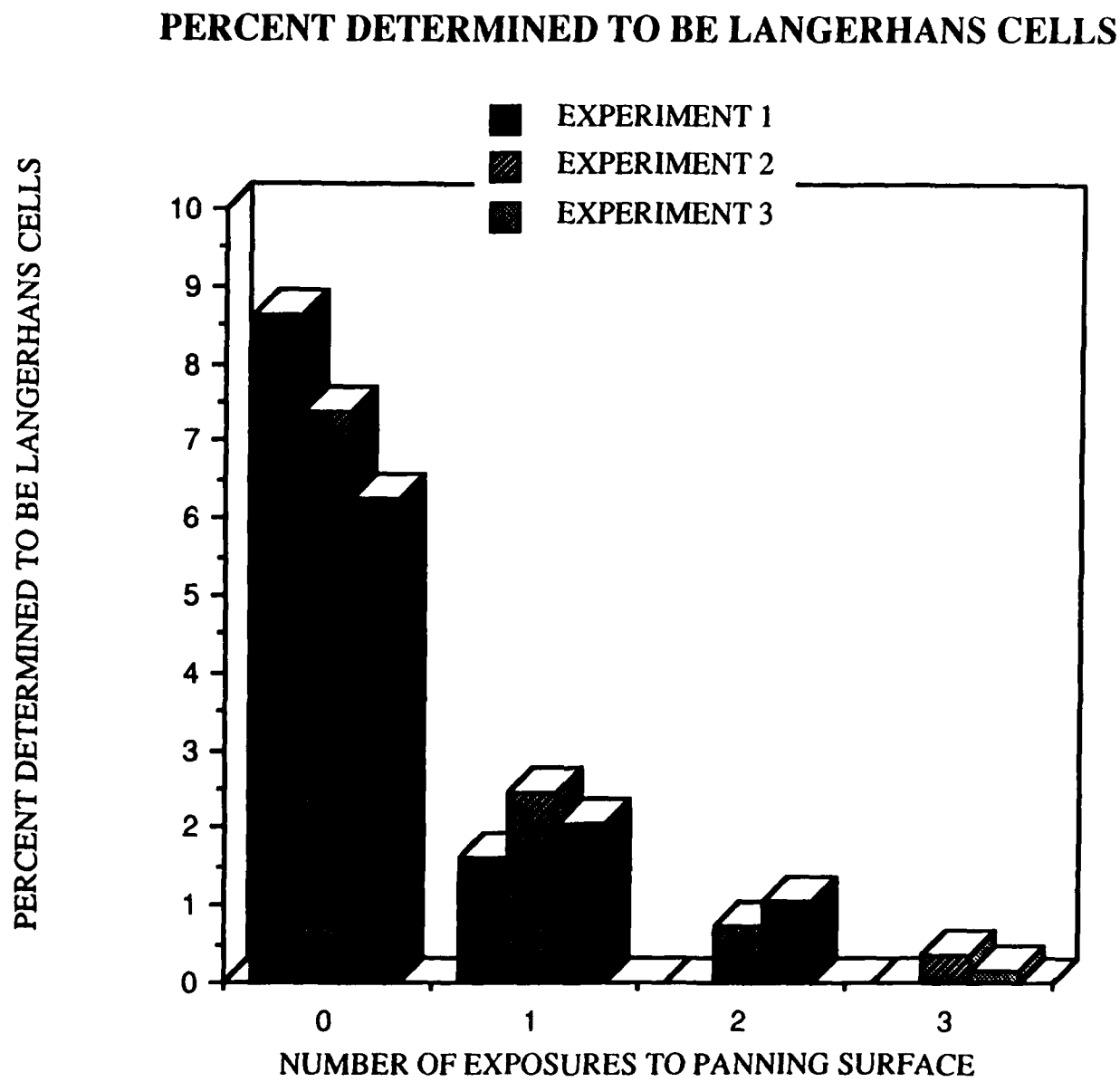
PERCENT DETERMINED TO BE LANGERHANS CELLS
EXPERIMENT 1
EXPERIMENT 2
EXPERIMENT 3
PERCENT DETERMINED TO BE LANGERHANS CELLS
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
NUMBER OF EXPOSURES TO PANNING SURFACE

FIGURE 2a

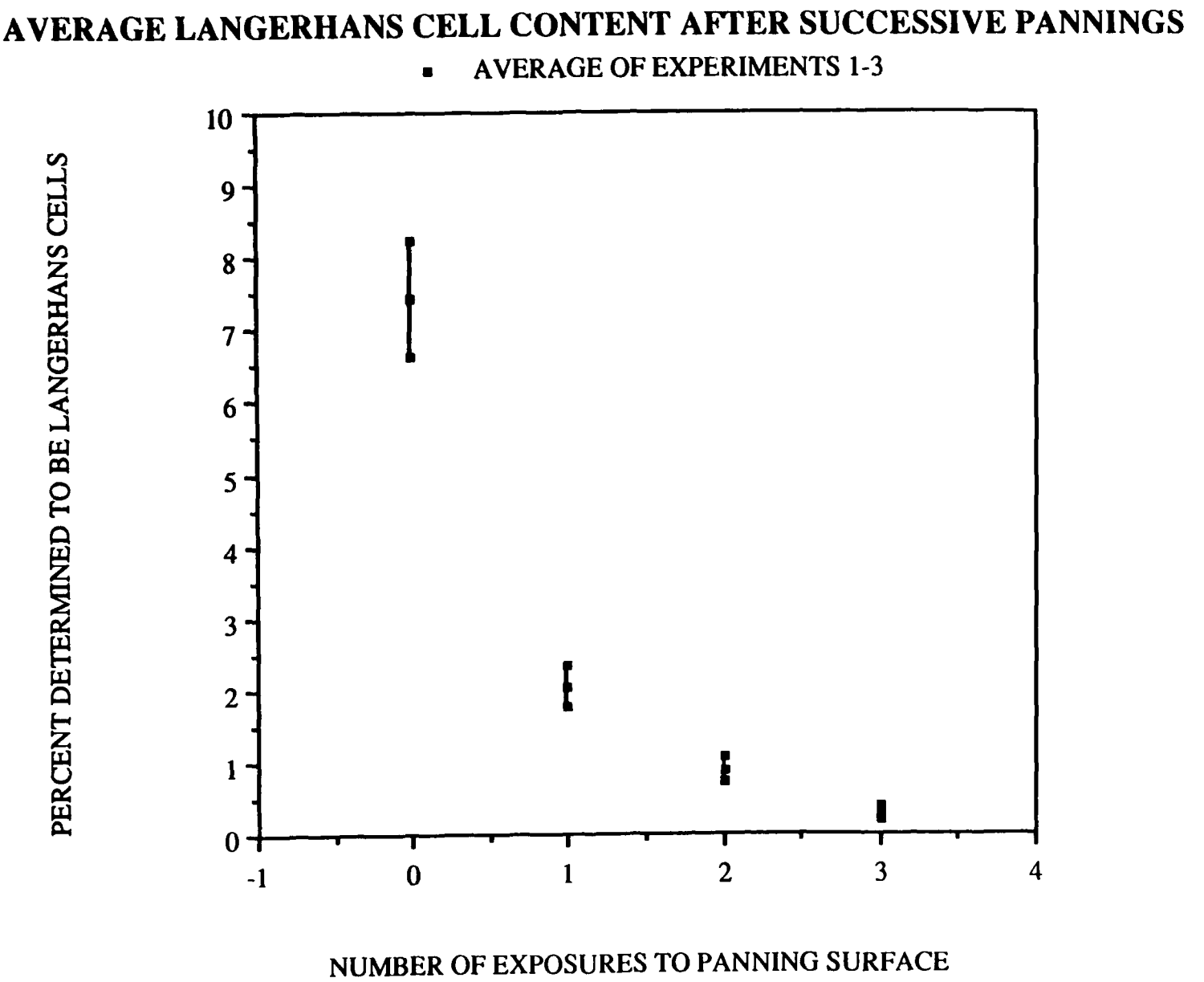
AVERAGE LANGERHANS CELL CONTENT AFTER SUCCESSIVE PANNINGS
AVERAGE OF EXPERIMENTS 1-3
PERCENT DETERMINED TO BE LANGERHANS CELLS
10
9
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
4
NUMBER OF EXPOSURES TO PANNING SURFACE

FIGURE 2b

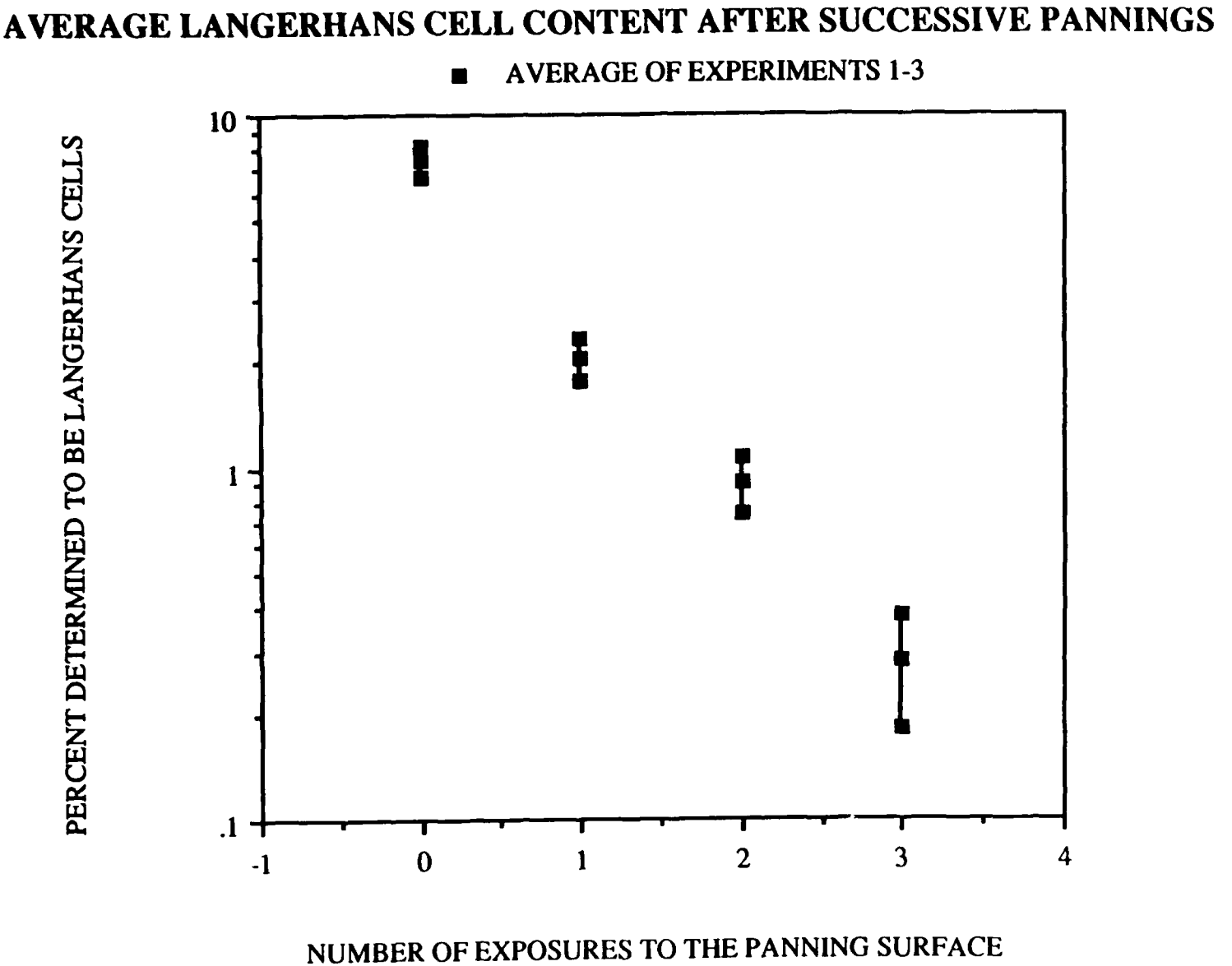
AVERAGE LANGERHANS CELL CONTENT AFTER SUCCESSIVE PANNINGS
AVERAGE OF EXPERIMENTS 1-3
PERCENT DETERMINED TO BE LANGERHANS CELLS
10
1
.1
-1
0
1
2
3
4
NUMBER OF EXPOSURES TO THE PANNING SURFACE

FIGURE 3

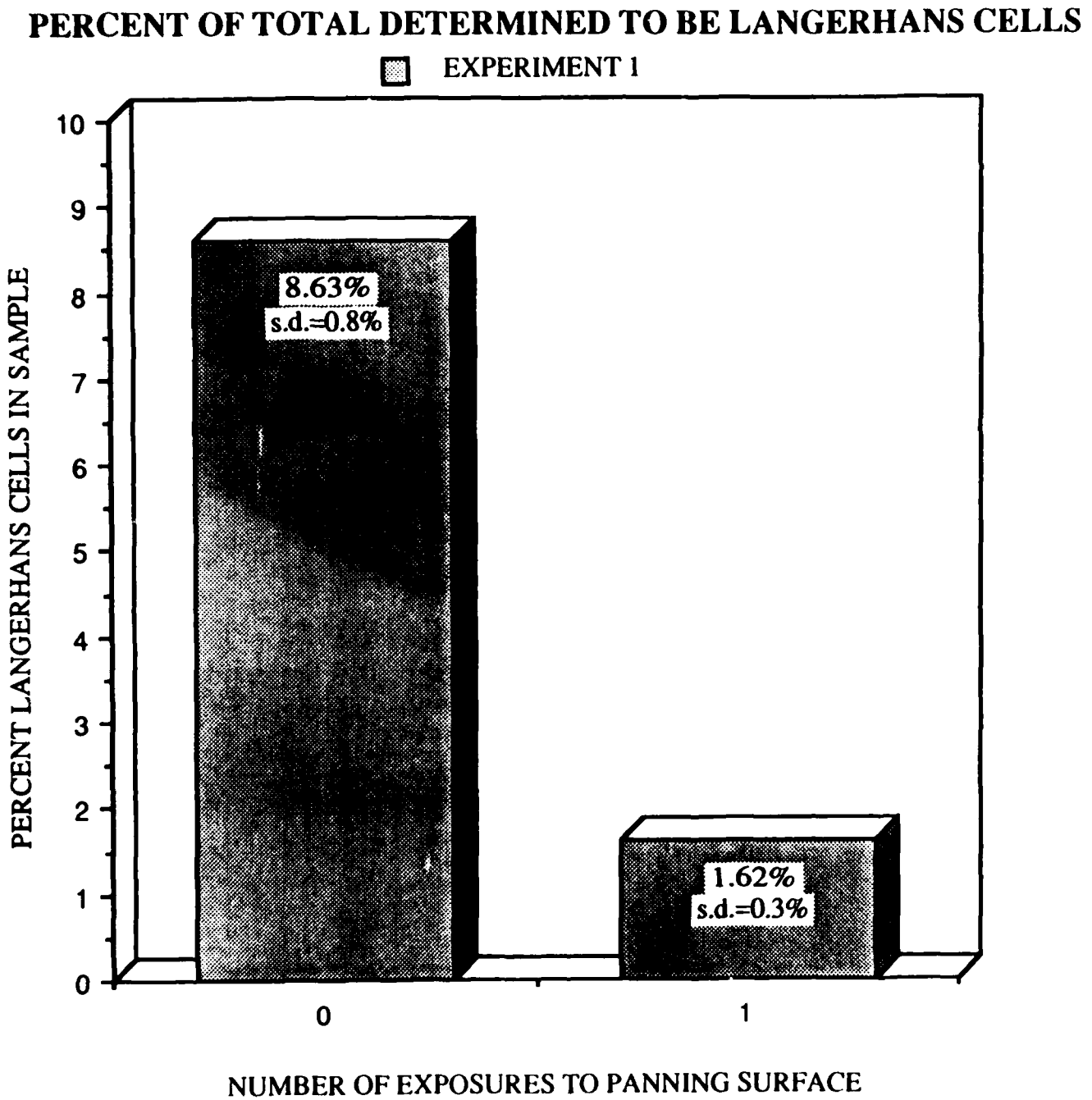
PERCENT OF TOTAL DETERMINED TO BE LANGERHANS CELLS
EXPERIMENT 1
PERCENT LANGERHANS CELLS IN SAMPLE
10
9
8
7
6
5
4
3
2
1
0
8.63%
s.d.=0.8%
1.62%
s.d.=0.3%
0
1
NUMBER OF EXPOSURES TO PANNING SURFACE

FIGURE 4

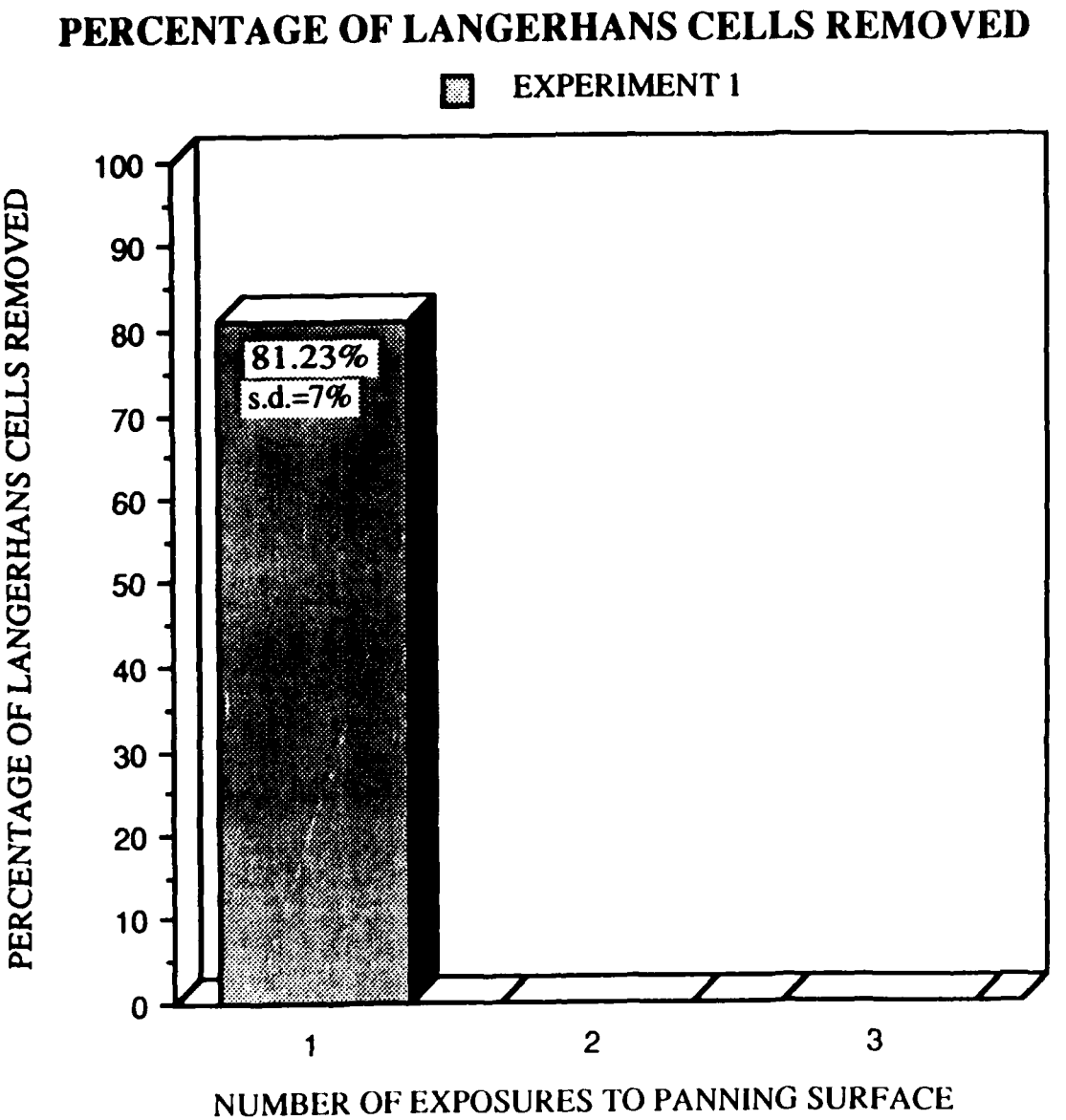
PERCENTAGE OF LANGERHANS CELLS REMOVED
EXPERIMENT 1
PERCENTAGE OF LANGERHANS CELLS REMOVED
100
90
80
70
60
50
40
30
20
10
0
81.23%
s.d.=7%
1
2
3
NUMBER OF EXPOSURES TO PANNING SURFACE

FIGURE 5

PERCENT OF TOTAL DETERMINED TO BE LANGERHANS CELLS

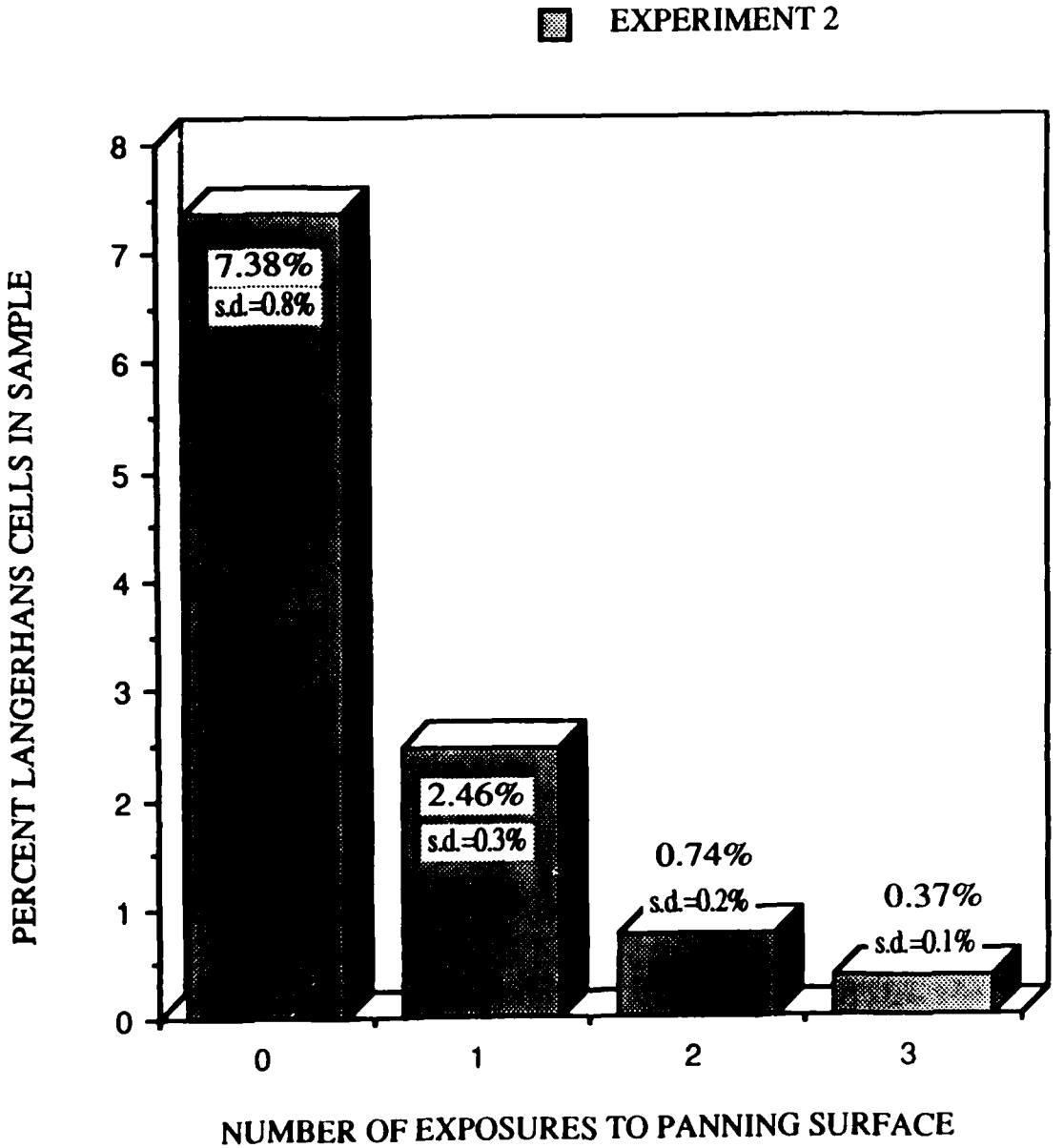

FIGURE 6

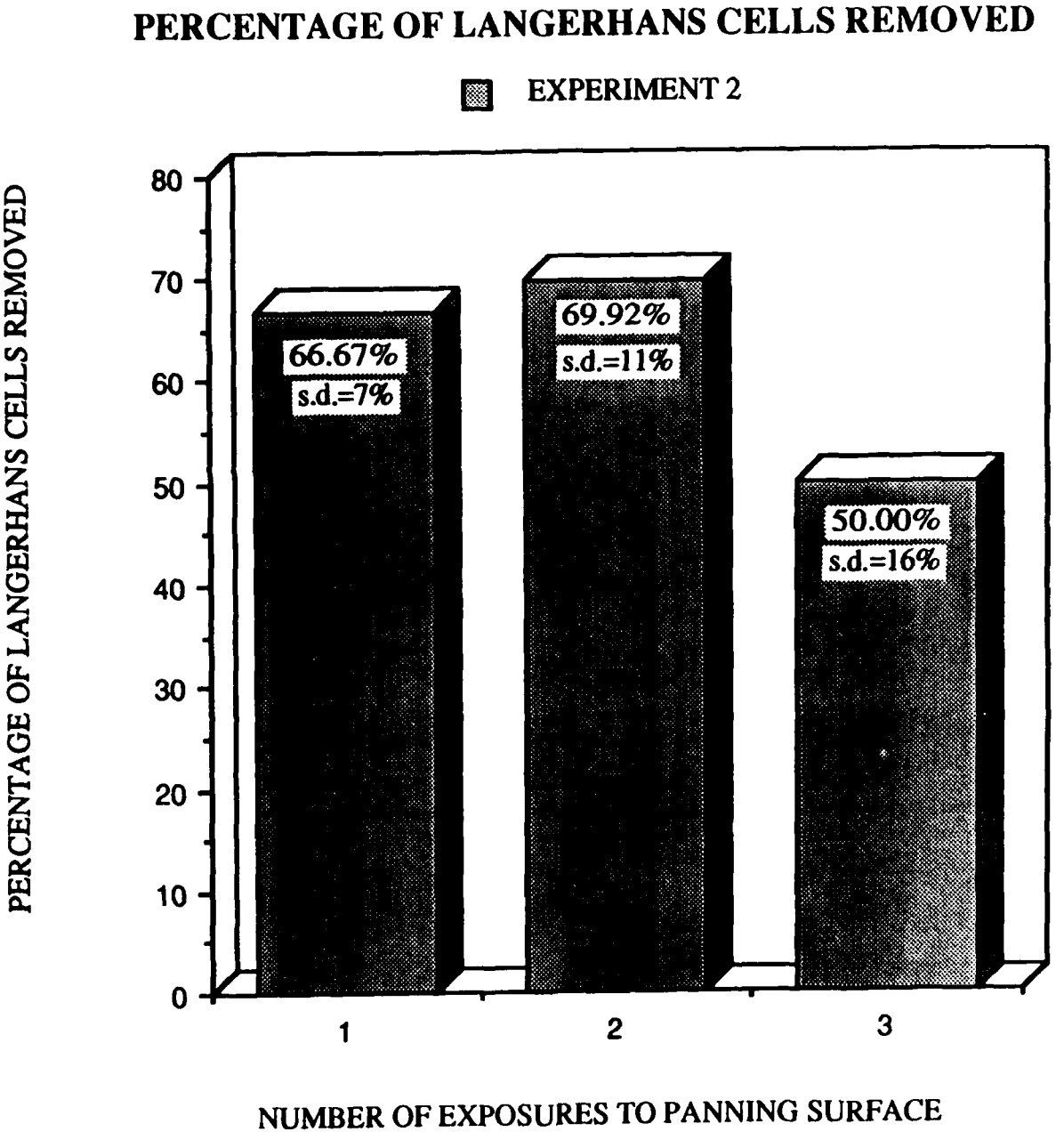
PERCENTAGE OF LANGERHANS CELLS REMOVED
EXPERIMENT 2
PERCENTAGE OF LANGERHANS CELLS REMOVED
80
70
60
50
40
30
20
10
0
66.67%
s.d.=7%
69.92%
s.d.=11%
50.00%
s.d.=16%
1
2
3
NUMBER OF EXPOSURES TO PANNING SURFACE

FIGURE 7

PERCENT OF TOTAL DETERMINED TO BE LANGERHANS CELLS

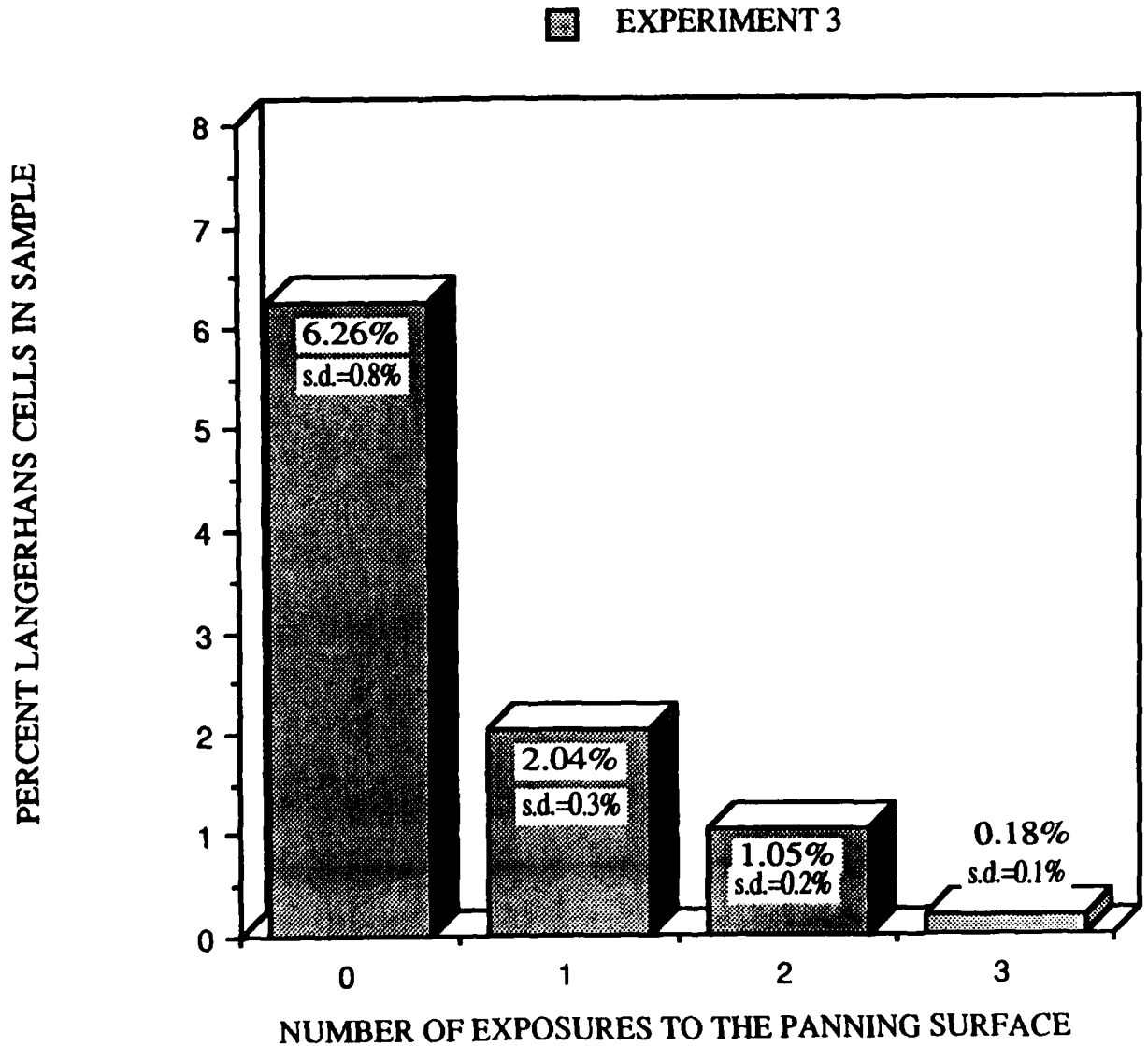

FIGURE 8

PERCENTAGE OF LANGERHANS CELLS REMOVED