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Invasiveness of *Pseudomonas aeruginosa* and Its Role in Diversity of Pseudomonal Infectious Diseases

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*Pseudomonas aeruginosa* causes both invasive (bacteremic) and chronic non-invasive infections in several organs, resulting in the diversity of infectious diseases. When Madin-Darby canine kidney (MDCK) monolayers were infected with clinical isolates of *P. aeruginosa*, significantly ($P < 0.001$) more blood (30 of 32, 93.7%) than respiratory (25 of 45, 54.4%) isolates penetrated the epithelial cell monolayers by 3 h. Only eight (4 blood and 4 respiratory) isolates were cytotoxic and possessed *exoU*, and passed through the monolayer following epithelial cell death associated with release of lactose dehydrogenase and a marked drop in transepithelial electrical resistance. Thus, invasiveness was usually independent of cytotoxicity. The capacity to penetrate epithelial cells appears to be a critical determinant of invasiveness in susceptible hosts and may be controlled by unknown unique genes. In addition, such invasion determinant(s) are thought to be predominantly exported by *P. aeruginosa* via MexAB-OprM, which is one of multi-drug resistant (MDR) efflux systems. Hence, MDR efflux systems in *P. aeruginosa* might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents.

**Key Words:** *Pseudomonas aeruginosa*, invasiveness, cytotoxicity, multi-drug resistant efflux system

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**Introduction**

*Pseudomonas aeruginosa* is a versatile Gram-negative bacterium that is an important pathogen in patients with compromised immunity, including those with cystic fibrosis (CF), neutropenia, thermal burns, and AIDS. *P. aeruginosa* is capable of establishing both chronic and acute infections. In patients with CF or diffuse panbronchiolitis (DPB), *P. aeruginosa* is the most predominant pathogen, causes chronic infection, and is difficult to be eliminated but usually does not invade the bloodstream. This organism, however, is invasive in other patient groups and causes bacteremia associated with high mortality rates, particularly in those with neutropenia. *P. aeruginosa* also causes several other infections in various organs. Thus, this organism develops a diversity of infectious diseases (Fig. 1).

**Figure 1.** A diversity of infectious diseases caused by *P. aeruginosa*.

**Bacterial infections**

- Diffuse panbronchiolitis
- CF

**Respiratory infections**

- Acute exacerbation
- Chronic persistent infections (i.e., DPB, CF)
- Pneumonias
  - Necrotizing, ventilator-associated, terminal

**Urinary infections**

- Endogenous (primary) septicemia (i.e., leukemia, burn)
- Secondary septicemia
- Meningitis
- Myelitis

**Skin/soft tissue infections**

- DMARD efflux systems in *P. aeruginosa* might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents.
Indeed, human blood isolates of \textit{P. aeruginosa} cause lethal endogenous bacteremia in neutropenic mice induced by cyclophosphamide\cite{1, 2} but human respiratory isolates do not\cite{3}. The animal model used in this study (Fig. 2) reflects the important pathophysiological steps in authentic human infections, including bacterial colonization and invasion. Clinical blood isolates of \textit{P. aeruginosa} have the ability to produce larger amounts of exoproducts, including exotoxin A and alkaline protease, in vitro, than the strains isolated from respiratory tract\cite{4, 5}. The blood isolates penetrate human intestinal Caco-2 epithelial cell monolayers to a greater degree than do the respiratory isolates\cite{6}. These phenotypic findings suggest that blood isolates carry virulence determinants which confer the invasive phenotype. Despite evidence of putative virulence determinants in \textit{P. aeruginosa}, none have been characterized yet. This is in contrast to what is known about other invasive bacteria such as \textit{Yersinia, Listeria monocytogenes, Shigella, Salmonella}, and \textit{Escherichia coli} (reviewed in reference 8). In this article, the author briefly reviews the recent findings in invasiveness of \textit{P. aeruginosa}, focusing on the own data in this field.

**Figure 2.** Endogenous \textit{P. aeruginosa} bacteremia model in mice. Mice were given orally \textit{P. aeruginosa} in drinking water at the concentration of 10⁷ CFU/ml between days 1 and 4. Mice were given intraperitoneally 200 mg of ampicillin per kg between days 1 and 4, and 200 mg of cyclophosphamide per kg on days 6, 9, and 12. If the isolate inoculated is invasive, the pathogen causes endogenous gut-derived septicemia in mice.

**Two phenotypes of \textit{P. aeruginosa}: invasive phenotype and cytotoxic phenotype**

Recently, it has been reported that \textit{P. aeruginosa} strains can be differentiated into two groups: i) strains with a cytotoxic phenotype such as PA103; and ii) strains with an invasive but non-cytotoxic phenotype such as PAO1\cite{7, 8}. In these studies, the invasiveness of \textit{P. aeruginosa} has been evaluated in Madin-Darby canine kidney (MDCK) cells and corneal epithelial cells by gentamicin survival assay\cite{9, 10}. Recently, it has also been reported that \textit{P. aeruginosa} strain PA103 carries \textit{exoU}, while PAO1 lacks it\cite{11}. \textit{ExoU} encodes a 70-kDa protein (\textit{ExoU})\cite{12}, which is identical to \textit{PepA}\cite{13}. It is a cytotoxin secreted by a type III system (reviewed in reference 13) and exerts an acute cytotoxic effect on epithelial and phagocytic cells. \textit{ExoU} is a possible candidate for the gene controlling invasiveness; however a correlation between its presence and the clinical sources of different \textit{P. aeruginosa} strains has not been investigated.

**Establishment of MDCK cell monolayer system and penetration of representative isolates**

We have developed an MDCK cell monolayer penetration assay\cite{14, 15} as an in vitro screening system for invasiveness of \textit{P. aeruginosa}\cite{16} (Fig. 3). When grown on permeable supports, polarized MDCK cells establish a monolayer with tight junctions and high transmonolayer electrical resistance, akin to Caco-2 cells\cite{17}. Monolayers of polarized MDCK cells were prepared in Transwell filter units containing 0.33-cm² porous filter membranes (3.0-µm pores) in 24-well tissue culture plates. Monolayers were infected with bacteria by adding 5 µl (ca. 3.5 x 10⁷ CFU) freshly grown bacteria cultured in L-broth overnight at 37°C.

First, \textit{P. aeruginosa} PAO1\cite{18} and PA103\cite{19} were examined in this system. \textit{Salmonella typhimurium} SL1344\cite{20} was used as a positive control. Noninvasive rabbit enterotoxigenic \textit{E. coli} strain RDEC-1\cite{21}, which is positive for oxidation of lactose, was used as a negative control and as an internal control of monolayer integrity, since it does not penetrate the monolayer unless the tight junctions are disrupted by Ca²⁺-free medium\cite{22, 23}. Some clinical isolates of \textit{P. aeruginosa} included five blood isolates and five respiratory isolates, which were previously evaluated in a murine model of endogenous septicemia\cite{24} and Caco-2 cell monolayer penetration assay\cite{25}, were also evaluated. In some experiments, an equal number of \textit{E. coli} RDEC-1 was added with \textit{P. aeruginosa} isolates or \textit{S. typhimurium} SL1344 at the same time. In these co-infection assay,
basolateral medium was plated on MacConkey agar (Quelab, Montreal, Quebec, Canada) to distinguish lactose-positive *E. coli* colonies from others.

The capacity of *P. aeruginosa* PAO1 and PA103 to penetrate through MDCK cell monolayers was compared with that of *S. typhimurium* SL1344, and *E. coli* RDEC-1 (Fig. 4). PAO1 was detected in the basolateral medium by 3 h after inoculation, as was *S. typhimurium* SL1344, whereas *P. aeruginosa* strain PA103 was not detected until 6 h. *E. coli* RDEC-1 was not detected by 12 h, and did not appear in the basolateral medium until at least 24 h after inoculation. There was no difference in growth rate between *P. aeruginosa* PAO1 and PA103 (data not shown).

Using the 10 clinical isolates a correlation between the data using MDCK and Caco-2 cells was examined. Times when bacteria appeared in the basolateral medium, and recovery of bacteria from the basolateral medium at 3h and 6h after inoculation correlated well (r = 0.911, 0.950, and 0.874, respectively) between the two cell lines (data not shown).

**Figure 4.** Bacterial penetration of *P. aeruginosa* PAO1 (■) and PA103 (■), *S. typhimurium* SL1344 (□), and *E. coli* RDEC-1 through MDCK cell monolayers (reference 16). Bacteria were inoculated at 3.5 x 10^8 CFU/well to the apical surfaces of MDCK cell monolayers. The assay was performed in triplicate, and results are expressed as an average ± standard deviation. Noninvasive *E. coli* RDEC-1 could not be recovered from the basolateral medium up to 12 h after inoculation.

Penetration of *P. aeruginosa* clinical isolates through MDCK cell monolayers.

A total of 77 clinical isolates of *P. aeruginosa*, including 32 blood and 45 respiratory isolates were evaluated using the MDCK cell monolayer system described above. *P. aeruginosa* isolates were divided to four groups based on clinical site of isolation and their capacity to penetrate through MDCK cell monolayers (table 1). Of 32 blood isolates of *P. aeruginosa*, 30 (93.7%) were detected in the basolateral medium by 3h, as was *P. aeruginosa* PAO1 and *S. typhimurium* SL1344. A total of 24 of 45 respiratory isolates of *P. aeruginosa* (54.4%) were detected in the basolateral medium by 3h; this percentage was significantly lower than among blood isolates (P < 0.001). The average number of bacteria detected in the basolateral medium at 3 h in blood isolates was significantly greater than the value of respiratory isolates (3.26 ± 5.05 x 10^3 versus 1.06 ± 1.03 x 10^3 CFU, P < 0.05).

**Phenotypes of clinical isolates of *P. aeruginosa*.**

Serum sensitivity of *P. aeruginosa* strains was evaluated as previously described. *P. aeruginosa* strains P1 and M2 were used as serum-sensitive and -resistant controls, respectively. The motility of *P. aeruginosa* isolates was assessed by the diameter of colonial spreading in soft L-agar containing 0.3% agar as previously reported. The presence of surface functional pili on *P. aeruginosa* strains was confirmed by plating a 5 μl drop of culture supernatant containing 4 x 10^9 particles of bacteriophage PO4 onto a freshly spread lawn of bacteria made on L-broth agar as previously reported.

Significantly greater numbers of serum-sensitive and nonmotile phenotypes were found among respiratory isolates than blood isolates (P < 0.001 and P < 0.05, respectively), and in respiratory isolates that were not able to penetrate MDCK cell monolayer than other groups as defined in table 1 (P < 0.001 and P < 0.005, respectively). Eight respiratory isolates, which were serum-resistant and also motile could not penetrate the monolayer by 3h. There was no difference in PO4 phage sensitivity between blood and respiratory isolates (P = 0.559), and among the four groups as defined in table 1 (P = 0.076). Most clinical blood isolates penetrated MDCK cell monolayers by 3 h, suggesting that they possess virulence determinants capable of inducing bacteremia. It appears that serum-resistance and motility may be necessary but not sufficient, since several motile serum-resistant respiratory isolates failed to pass through the monolayer. Although piliation in *P. aeruginosa* is also thought to be important in the colonization of the CF airway and in the establishment of other infections, its significance in the penetration of isolates through MDCK cell monolayer was not clearly demonstrated in this study (table 1).
Table 1. Penetration of clinical isolates of *P. aeruginosa* through MDCK cell monolayers, their phenotypes, and possession of exoU (reference 16).

<table>
<thead>
<tr>
<th>Origin</th>
<th>Total No.</th>
<th>Capacity to penetrate MDCK cell monolayers by 3 h</th>
<th>Mucoid</th>
<th>Serum-sensitive</th>
<th>Nonmotile</th>
<th>PO4-resistant</th>
<th>Cytotoxic</th>
<th>exoU gene positive by:</th>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>0</td>
<td>1</td>
<td>1</td>
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<td>3</td>
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<td>Respiratory tract</td>
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<td>3</td>
<td>11</td>
<td>7</td>
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* a Significantly greater numbers of blood than respiratory isolates penetrated MDCK cell monolayer by 3 h ($P < 0.001$).

b Significantly greater number of serum-sensitive isolates were found in respiratory isolates than blood isolates ($P < 0.001$), and in respiratory isolates which did not penetrate MDCK cell monolayer ($P < 0.001$).

c Significantly greater number of nonmotile isolates were found in respiratory isolates than blood isolates ($P < 0.05$), and in respiratory isolates which did not penetrate MDCK cell monolayer ($P < 0.005$).

Detection of *exoU* gene and its role in cytotoxicity.

To quantify cytotoxicity of *P. aeruginosa* isolates on the MDCK cell monolayers, the concentration of lactose dehydrogenase (LDH) released from the MDCK cells into the medium in the filter units was measured. Triton X-100 was used as a positive control to destroy the cells and make LDH release. First, the LDH level in both the apical and basolateral medium was measured for monolayers incubated with either PAO1 or PA103 at several time points after infection (Fig. 5). Although LDH was not detected by 1 h, LDH was released into the apical medium from the MDCK monolayer inoculated with PA103 by 3 h. When the MDCK cell monolayer was infected with PAO1, LDH was not detected until at least 6 h and not in substantial levels until 10 h. LDH was not detected in the basolateral medium until 6 h and 10 h after infection with PA103 and PAO1, respectively. Cytotoxic strains were defined as those which caused statistically significant levels of LDH release from MDCK cell monolayers in apical medium by 6h, as compared with spontaneous LDH release. Using this definition, 8 of 77 clinical *P. aeruginosa* isolates (10.4%) were cytotoxic. These 8 cytotoxic isolates included four blood isolates (B22, B24, B34, and B39) and four respiratory isolates (S33, S36, S45, and S50). There was no significant difference in cytotoxicity between blood and respiratory isolates ($P = 0.609$).

The *exoU* gene was detected from *P. aeruginosa* isolates by both PCR and dot blot hybridization analysis. Chromosomal DNA was purified from the isolates as previously described. The oligonucleotide primers used for PCR detection of the *exoU* gene were designed from the sequence previously published (11, accession number U97065) for this study as follows; sense, 5'-TAG AAC GCC TAT TGC GCG-3'; antisense, 5' CTC GAG CTG CAG CAT TTC-3'. PCR was performed in a final volume of 25 μL containing 20 ng of *P. aeruginosa* DNA, 20 pmol each primer, 250 μM
each deoxynucleoside triphosphate, and 1 U of *Taq*
polymerase in 20 mM Tris-HCl (pH 8.4), 50 mM KCl,
and 1.5 mM MgCl₂, as follows: 30 cycles of 1 min at
94 °C, 1 min at 58 °C, and 1 min at 72 °C, followed by
final extension at 72 °C for 10 min. A DNA probe for
dot blot hybridization was prepared by amplification
of a 572 bp fragment of the exo*U* gene from *P.
aeruginosa* PA103 by PCR as described above and
was simultaneously labelled with digoxigenin-11-
uridine-5'-triphosphate (DIG-dUTP). Approximately 1
μg of DNA in TE from each of the *P. aeruginosa*
isolates was filtered under vacuum onto positively
charged nylon membranes by using a 96-well dot-
blot apparatus. The filters were hybridized with the
DIG-labelled exo*U* gene fragment and were devel-
oped by chemiluminescence detection as previously
described.

Figure 6 shows LDH levels in apical medium for the
cytotoxic isolates together with isolates showed a
slight but not statically significant level of LDH (S31,
S32, S35, and S56). The isolates positive for exo*U*
are also indicated in figure 6. The expected 572 bp frag-
ment of exo*U* was detected by PCR from 8 isolates in-
cluding blood isolates B22, B24, B34, and B39, and res-
piratory isolates S33, S36, S45, and S50 (table 1 and
Fig. 6). All these isolates were also positive for exo*U*
by dot blot hybridization (table 1 and Fig. 7). There
were no strains that showed discrepancies in exo*U*
gene detection between PCR and dot blot hybridiza-
tion. Figure 6 also shows that typical invasive iso-
lates, including PA01 and blood isolates B15, B16, and
B51, and non-invasive respiratory isolates such as S3
and S40 were non-cytotoxic and also were negative for
exo*U*.

In our study, 8 of 77 clinical isolates of *P. aeruginosa*
showed significant cytotoxicity towards MDCK cells
and all of these isolates carried exo*U* (table 1, figure
6). To our knowledge, this is the first report of the
frequency of exo*U* in clinical isolates. It has been re-
ported that *P. aeruginosa* isolates can be differenti-
ated into two groups as determined by cytotoxic or inva-
sive phenotype, as previously described. In the cur-
rent study, we could further differentiate clinical iso-
lates of *P. aeruginosa* into four groups as follows: i)
invasive and lack exo*U* (non-cytotoxic), ii) noninvasive
and carry exo*U* (cytotoxic), iii) invasive and carry exo*U*
(cytotoxic), and iv) noninvasive and lack exo*U* (non-
cytotoxic). Most blood isolates (27 of 32, 84.5%) and
fewer respiratory isolates (22 of 45, 48.9%) belonged to
the first group. One blood (B34) and two respiratory
(S33 and S50) isolates belonged to the second group.
Blood isolates, B22, B24, and B39, and respiratory iso-
lates S36 and S45 belonged to the third group. In
these isolates, B24, which was nonmotile, showed very
low penetration ability (< 5% of value of PA01), while
B22 showed marked penetration ability and was de-
tected from basolateral medium faster than PA01
data not shown). One blood isolate (B38) and 19 of 45
(42.2%) respiratory isolates belonged to the fourth
group. This group included all mucoid, and several se-
rum-sensitive, and nonmotile isolates. However, seven
serum-resistant and also motile respiratory isolates be-
longed to this group.

![Figure 6](image1.jpg)

**Figure 6.** LDH released from MDCK cells at 6 h after inocu-
lation and exo*U* gene detection in various *P. aeruginosa*
isolates. Cytotoxicity was evaluated by measuring LDH release from
the cells. Exo*U* was detected by both PCR and dot blot hy-
dridization. □ indicate isolates showed statistically significant
LDH release (*P* < 0.05), and □ indicate isolates showed not
statistically significant LDH release compared to spontaneous
LDH release from monolayers without infection (*P* > 0.1). All
isolates carrying exo*U* showed significant cytotoxicity (*P* <
0.05).

![Figure 7](image2.jpg)

**Figure 7.** Detection of exo*U* by dot blot hybridization (upper)
and PCR (lower). Hybridization was performed by using a digoxigenin-labelled
exo*U* fragment incorporated during PCR for *P. aeruginosa*
PA103, PA103, B22, B39, S36, and S50 were positive for exo*U*
by both methods. Invasive non-cytotoxic isolates, PA01, B15,
and B16, noninvasive non-cytotoxic isolates S3 and S40 were
negative for exo*U*. Four additional clinical isolates (B24, B34,
S33, and S45) were also positive for the gene by both meth-
ods (text, table 1, and figure 6).
Influence of *P. aeruginosa* infection on epithelial cell damages.

Transmonolayer electrical resistance (TER) was measured with a Millicell-ESR apparatus at sequential timed intervals.

Figure 8 shows the mean values of TER for cytotoxic isolates, PA103, S50, and B22, and non-cytotoxic isolates, PAO1, B15, and B16, with those of *S. typhymurium* SL1344 and *E. coli* RDEC-1. When MDCK cell monolayers were inoculated with *E. coli* RDEC-1, TER decreased slowly over time. The changes in TER for non-cytotoxic isolates was quite similar to that for *S. typhymurium* SL1344. TER for cytotoxic isolates were significantly lower than those for non-cytotoxic isolates between 4 and 12 h (*P* < 0.05).

When either *P. aeruginosa* PAO1 or B16 was added simultaneously to the apical medium with *E. coli* RDEC-1, *P. aeruginosa* alone was detected in the basolateral medium at 3 h, while *E. coli* RDEC-1 did not appear until 10 h after inoculation (data not shown). Similar results were obtained when the monolayer was infected with either *P. aeruginosa* B15 or B51 together with *E. coli* RDEC-1 (data not shown). In contrast, when cytotoxic *P. aeruginosa* strains PA103 or S50, and B22 were each inoculated together with *E. coli* RDEC-1, RDEC-1 was detected at the same early time as the *P. aeruginosa* isolates.

The co-infection study clearly revealed that cytotoxic isolates passed through MDCK cell monolayer by disrupting the monolayer, while non-cytotoxic isolates, such as PAO1, B15, B16, and B51, did not markedly affect monolayer integrity. The results in TER study and LDH assay also supported this. Consequently cytotoxic clinical isolates and PA103 are thought to pass through the monolayer following monolayer disruption due to intoxication.

Role of multidrug efflux systems in the invasiveness of *P. aeruginosa*

*P. aeruginosa* is intrinsically resistant to conventional penicillins and cephalosporins due to its low outer membrane (OM) permeability coupled to the production of an inducible chromosomal β-lactamase, which hydrolyzes these β-lactams and can become mutationally resistant to even newly-developed anti-pseudomonal agents. In addition to the recent emergence of metallo-β-lactamase producing *P. aeruginosa*, multidrug resistant (MDR) efflux systems are becoming recognized as important antimicrobial resistance mechanisms for this organism. To date, four resistance-nodulation-division (RND) MDR efflux systems have been well characterized in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY/OprM (reviewed in reference 42). More recently, an additional system, MexJK/OprM has been reported.

Recently, certain studies have suggested a relationship between efflux and virulence in *P. aeruginosa*. For instance, it has been reported that MexAB-OprM exports homoserine lactones which are involved in quorum sensing (cell-to-cell signaling) and consequent regulation of the expression of a variety of virulence determinants. However, the role of efflux systems in specific clinical pathogenesis of *P. aeruginosa*, including invasiveness, has not been determined. To gain a better understanding the role of efflux in bacterial pathogenesis, the invasiveness of *P. aeruginosa* PAO1 and its efflux mutants was evaluated using in vitro MDCK epithelial cell monolayer penetration. Virulence was also investigated in a murine model of...
endogenous *P. aeruginosa* bacteremia.

Both the parent WT strain K767 and strain K1521 (ΔmexCD-oprJ) penetrated MDCK monolayers by 3 h, whereas K1119 (ΔmexAB-oprM) was not detected in the basolateral medium until 6 h after infection (Fig. 9). Strains K767 (WT) and K1521 (ΔmexCD-oprJ) induced lethal endogenous septicemia in mice, whereas strain K1119 (ΔmexAB-oprM) failed to kill any mice (*P* < 0.0001, Fig. 10). To confirm the role of mexAB-oprM in the expression of invasiveness, the ΔmexAB-oprM deletion strain K1119 was complemented with mexAB-oprM by introducing plasmid pRSP17 carrying the genes and examined with strains K767 (WT), K1119 (ΔmexAB-oprM), and K1119/pRK415 (plasmid control). K1119/pRSP17 (mexAB-oprM complemented strain) showed the invasiveness equivalent to WT in both in vitro (Fig. 11A) and the animal model (Fig. 11B), while plasmid control strain K1119/pRK415 had the same phenotype, which was compromised in its capacity to penetrate MDCK monolayer (Fig. 10A, *P* < 0.0001) and to kill leukopenic mice (Fig. 11B, *P* < 0.0001) as K1119 (AmexAB-oprM).

These findings strongly suggest that invasion determinant(s) are predominantly exported by *P. aeruginosa* via MexAB-OprM. Hence, MDR efflux systems in *P. aeruginosa* might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents. It seems practical for bacteria to utilize efflux systems to export virulence determinants and physiological products, as a physiological process.

**References**


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