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immunodeficiency

Monocyte Chemotaxis under Agarose: Defects in Atopic Disease, Aspirin Therapy, and Mucocutaneous Candidiasis

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Summary

Using Ficoll-Hypaque-separated cells, monocyte chemotaxis was measured by an agarose technique in patients with increased susceptibility to infection, with atopic dermatitis, and in individuals taking aspirin. *In vitro* effects of aspirin, hydrocortisone, aminophylline, ephedrine, and diphenhydramine were also studied. Significantly decreased chemotaxis was found in one 9-year-boy with severe mucocutaneous candidiasis and three of 22 patients with atopic dermatitis. In the atopic group of patients greater than 10 years of age, mean monocyte chemotaxis was significantly decreased from the age-matched control group. This decrease did not correlate with serum IgE levels, absolute blood eosinophil counts, or clinical symptom scores. Following aspirin ingestion, mean monocyte chemotaxis significantly decreased whereas neutrophil chemotaxis was unaffected. Using therapeutic concentrations, drug levels of aspirin and aminophylline *in vitro* caused greater than 35% inhibition of monocyte movement.

Speculation

Measurement of monocyte chemotaxis in immunodeficiency and atopic disease may uncover defects amenable to agents which stimulate cell movement.

Defective monocyte chemotaxis has been observed in a variety of diseases, including chronic mucocutaneous candidiasis (11, 27), Wiskott-Aldrich syndrome (1), Chediak-Higashi syndrome (12), atopic dermatitis (10, 25, 29), malignancy (28), and viral infections (16). Developmental monocyte chemotaxis defects have also been noted in normal newborns and young children utilizing endotoxin or zymosan-activated serum as the chemotactic factor (15, 31).

The majority of these studies used the Boyden chamber technique. A sensitive method of measuring chemotaxis under agarose, described by Cutler (6) and Nelson *et al.* (21), offers several advantages over the Boyden chamber method, including the need for fewer cells and less chemotactic fluid, disposable, inexpensive equipment, and direct *in situ* observation.

This report describes our experience using an adapted Cutler technique for determining monocyte and, in selected cases, neutrophil chemotaxis in patients with undue susceptibility to infection, atopic dermatitis, and in normal individuals taking aspirin. *In vitro* effects of several commonly used drugs were also studied.

MATERIALS AND METHODS

SUBJECTS

Thirty-three patients with repeated infections were studied. Fourteen had primary immunodeficiencies including chronic mucocutaneous candidiasis (5), severe combined immunodeficiency (1), congenital agammaglobulinemia (5), Job-Buckley syndrome (1), Nezelof syndrome (1), and DiGeorge syndrome (1). Five patients with recurrent respiratory infections of unknown cause, six children with cystic fibrosis, and eight adults with chronic renal failure prior to dialysis were also investigated.

We also studied 22 patients with atopic dermatitis ranging in age from 17 months to 24 years (mean age, 10.2 years). There were 16 males and 6 females with a mean duration of the dermatitis of 8.3 years. Sixteen of these patients had asthma, and 15 had allergic rhinitis. Concurrent treatments within one week of the study included corticosteroid creams (14 of 22), hydroxyzine (11 of 22), diphenhydramine (4 of 22), prednisone (1 of 22), Marax (ephedrine sulfate, theophylline, hydroxyzine, and ethyl alcohol) (2 of 22), and theophylline (1 of 22) (PO medications were withheld for at least 6 to 12 hr prior to testing). In addition to chemotaxis determination, total serum IgE by Phadebas RIST method, total absolute blood eosinophil counts by Pilot technique (22), and a clinical symptom score were determined. The latter, adapted from the scoring system of Rachelefsky *et al.* (23) was based upon eight criteria: (1) erythema; (2) vesiculation; (3) crusting; (4) excoriation; (5) lichenification; (6) pigmentation; (7) pruritus; and (8) sleep loss. Gradations were given to each criteria (0 to 4) depending on the number of body areas involved. A total score was determined which ranged from 0 (maximally severe disease) to 32 (no disease).

A third study group consisted of eight adult volunteers who ingested 3.9 g of aspirin daily for three days. Monocyte and neutrophil chemotaxis were measured pre- and postaspirin ingestion. Serum salicylate concentrations were measured by the UCLA clinical laboratory.

In vitro effects on monocyte chemotaxis were determined by adding varying concentrations of hydrocortisone sodium succinate (Solu-Cortef; The Upjohn Co., Kalamazoo, MI), ephedrine sulfate (Abbott Laboratories, North Chicago, IL), aminophylline ethylenediamine (Amersham/Searle Corp., Arlington Heights, IL), diphenhydramine (Parke, Davis and Co., Detroit, MI) and acetylsalicylic acid (Stayner Corp., Hayward, CA) to Ficoll-Hypaque separated mononuclear cells from normal adult volunteers.

Informed consent was obtained in accordance with the UCLA Human Subjects Protection Committee regulations.

CHEMOTAXIS METHOD

Mononuclear cells were obtained from heparinized blood (final heparin concentrations of 20 units/ml of blood) by density gradient separation using Ficoll-Hypaque with a specific gravity of 1.077 to 1.084. The usual yield was 2×10^6 cells for each millimeter of blood with approximately 70% of the cells lymphocytes and 30% mononuclear (MN) phagocytes. Cell percentages were further determined by Wright's stained cytocentrifuged preparations. After washing three times in Hanks' balanced salt solution, the cells were suspended in Roswell Park Memorial Institute medium (RPMI) with 5% fetal calf serum to a final concentration of 10^7 MN's/ml. Polymorphonuclear leukocytes were separated by sed-

imentation using 15% sterile Plasmagel according to the method of Forman and Stiehm (32). Serum samples were stored at −20°C until tested.

Monocyte chemotaxis was measured using a Cutler technique adapted by Klein *et al.* (15). In this assay, 1.5 ml of a 0.75% electrophoresis grade agarose in medium 199 with 10% fetal calf serum is plated in a 35-x 10-mm diameter disposable plastic Petri dish. Three wells, 4 mm in diameter and 2 mm apart, are made in the agarose plate with a template punch, and then the plugs are removed with suction. Ten μl of MN cells (10^7/ml) are added to the central well, 10 μl of chemotactic factor are added to the left well, and 10 μl of normal saline are added to the right well (Fig. 1). All assays are done in triplicate. The agarose plates are incubated at 37°C in a 5% CO_2 atmosphere for 18 hr. Counting is facilitated by using a microscope 5- x 5-mm eyepiece grid; this is aligned between the center well and a peripheral well to form a vertical column. Cells in both columns are counted using an × 10 magnification and expressed as the mean migrating cells per plate (cells migrating toward activated serum minus cells migrated towards saline). The agarose plates can be fixed by adding 1.5 ml of absolute alcohol to the plate for 4 hr. The agarose is then removed, and the plate is stained with Wright's stain.

Polymorphonuclear (PMN) chemotaxis was similarly measured but using cell suspensions of 2.5×10^6 cells/ml and an incubation period of 3 hr. Age-matched chemotaxis values for both monocytes and neutrophils were previously reported in our laboratory (15); day control determinations from normal adults were measured for each experiment.

The chemotactic factor for all assays was zymosan-treated serum. Three ml of human serum of a standard adult donor was added to 0.15 g of zymosan at 37°C for 30 min, centrifuged at 900 × *g* for 10 min, inactivated at 56°C for 30 min, and stored in aliquots at −20°C.

To assess serum inhibition of monocyte chemotaxis, undiluted patient's serum was incubated with equal portions of a MN cell suspension from a standard normal adult donor for 30 min, washed twice with Hanks' balanced salt solution, and resuspended in RPMI for the chemotaxis determination.

To assess possible cytotoxicity of drugs added to mononuclear preparations, Ficoll-Hypaque separated cells from four normal adults were washed twice with Hanks' balanced salt solution and suspended in RPMI with 2.5% fetal calf serum. Drugs and cells were mixed in the same concentrations as in the chemotaxis experiments and incubated in a 5% CO_2 incubator for 3 and 18 hr. Trypan Blue (0.4%) was added, and 200 cells were counted for dye exclusion.

Student's *t* test was used to analyze the difference between groups tested. Correlation coefficients were computed on an Olivetti Underwood Programma 101. In statistical comparisons, *P* values less than 0.05 are considered significant.

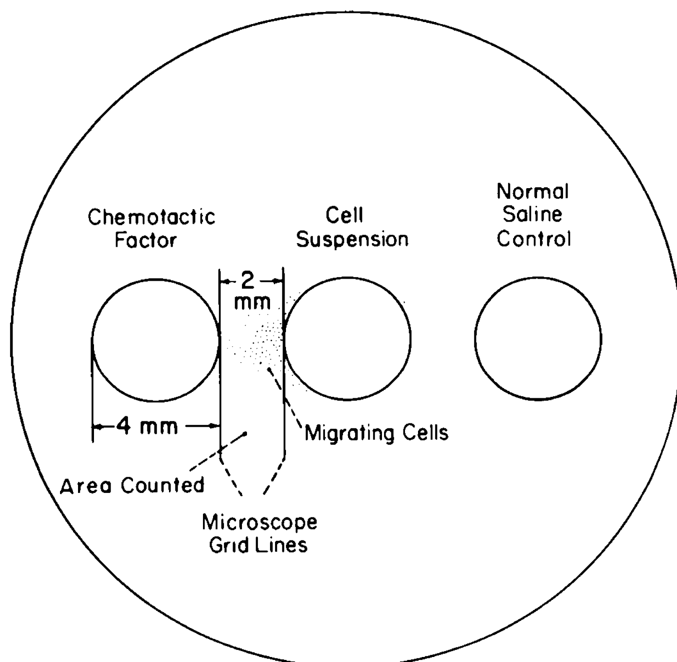

Fig. 1. Diagram of agarose method of chemotaxis performed in a 35-mm diameter plastic Petri dish. Migration occurs beneath the layer of agarose (15).

RESULTS

PATIENTS WITH INCREASED SUSCEPTIBILITY TO INFECTION

Of the 33 patients tested, one child had a persistent decrease from normal age-matched controls on nine separate occasions when he was not receiving parenteral antifungal medications (Table 1). This 9-year-old boy has severe chronic mucocutaneous candidiasis, bronchiectasis, failure to thrive, hypothyroidism, and recurrent otitis media and sinusitis. He has not responded to transfer factor, levamisole, or lymphocyte transfusions and requires repeated courses of IV amphotericin B or miconazole. He has been consistently anergic to *Candida albicans* 1/10 (Hollister-Stier Laboratories, Los Angeles, CA), SKSD 1000 μg/250 μm (Lederle Laboratories, Pearl River, N.Y.) and phytohemagglutinin 1 μg/0.1 ml (Burroughs Wellcome & Co, Research Triangle Park, N.C.). *In vitro*, he has decreased lymphocyte blastogenic response to *C. albicans* antigen and decreased macrophage inhibition factor production. Quantitative immunoglobulins, B- and T-cell enumeration, neutrophil chemotaxis, mixed lymphocyte culture reactivity, and *in vitro* phytohemagglutinin reactivity were normal. In addition to the chemotactic defect, *in vitro* monocyte killing of *C. albicans* was decreased on three separate occasions. Staphylococcal killing was normal.

PATIENTS WITH ATOPIC DERMATITIS

Mean monocyte chemotaxis values (± S.E.) of the atopic patient groups were compared with values from age-matched normal control groups (based on 86 normal individuals previously reported from our laboratory) (Ref. 15; Fig. 2). Mean values (± S.E.) for patient groups 1 month to 2 years (36 ± 27) and 3 to 5 years (68 ± 50) were not significantly different from controls (31 ± 6 and 71 ± 15, respectively). Although mean monocyte chemotaxis in the 6- to 10-year (125 ± 33) group was lower than that in controls, this decrease was not statistically significant. However, atopic patients 11 years and older as a group (123 ± 24) had significantly reduced mean values from controls (215 ± 12). No significant correlation was found between monocyte chemotaxis and serum IgE levels, blood eosinophil counts, or symptoms.

When individual patients were compared, 3 patients (Table 2) had monocyte chemotaxis values greater than 2 S.D.'s below age-matched controls (<70 cells migrated). Their eosinophil counts, serum IgE values, and clinical scores were not different from patients with normal monocyte chemotaxis.

Table 1. *Monocyte chemotaxis determinations in a patient with severe mucocutaneous candidiasis*

Date	Cells migrated/18 hr[1]
02/02/76	1
03/08/76	76
03/15/76	9
09/27/76	86
12/13/76	31
01/03/77	8
03/08/77	48
02/24/77	5
05/24/77	30

[1] Age-matched controls, 142 ± 64 (1 S.D.).

Sera from the 3 patients with depressed values inhibited normal monocyte chemotaxis 12, 26, and 43% (mean 27 ± 16) compared with mean-inhibitory activity from five random adult normal controls of 28 ± 6% (1 S.D., range, 22 to 36%.) The monocyte chemotaxis values of the controls were normal.

DRUG EFFECTS

Results of *in vitro* drug effects on monocyte chemotaxis are presented in Table 3. Concentrations of aminophylline (8) in the therapeutic range resulted in greater than 35% decrease from control levels (cells without added drug). Increasing concentrations of hydrocortisone, ephedrine, and diphenhydramine resulted in depression of chemotaxis. However, the inhibiting levels of

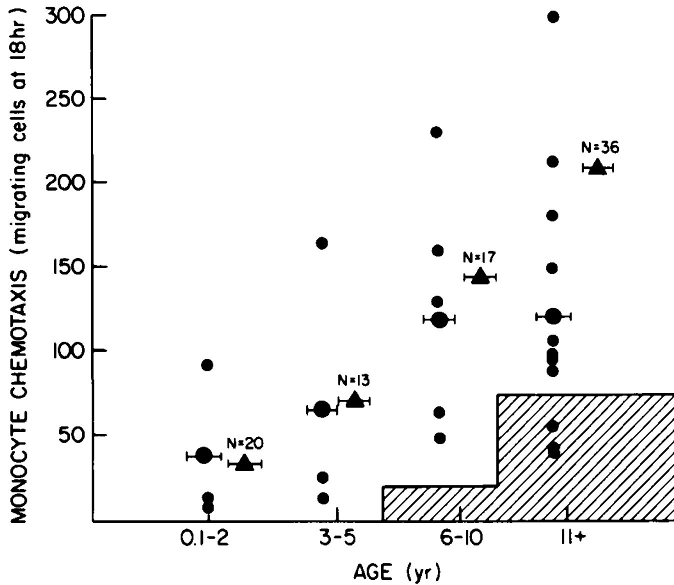

Fig. 2. Mean age-related monocyte chemotaxis values between atopic patients (●) and normals (▲). *N*, number of normals in each age group. *Shaded area*, values greater than 2 S.D. from normal (<70 for 11+ group; <14 for 6 to 10 group).

Table 3. *Drug inhibition of monocyte chemotaxis*

Drug/Concentration	No. of Donors	% inhibition
Ephedrine (μg/ml)	3	
50		0.30 ± 0.6[1]
100		39.0 ± 17.5
250		33.0 ± 30.0
1000		85.0 ± 15.6
Diphenhydramine (μg/ml)	3	
50		0.0 ± 0.0
100		62.3 ± 8.39
250		87.7 ± 11.0
1000		100.0 ± 0.0
Aminophylline (μg/ml)	7	
5		37.1 ± 33.4
10		46.3 ± 33.2
25		38.9 ± 28.8
125		37.0 ± 32.3
Hydrocortisone (μg/ml)	6	
2		2.4 ± 5.7
20		2.4 ± 5.4
50		35.3 ± 32.2
100		37.8 ± 26.4
250		44.3 ± 35.5
Aspirin (mg/dl)	4	
20		61.5 ± 15.5
50		72.8 ± 17.3

[1] Mean ± S.D.

Table 2. *Monocyte chemotaxis values, serum IgE concentrations, and eosinophil counts in patients with atropic dermatitis*[1]

Patients	Age (yr)	Sex	Monocyte Chemotaxis (cells migrated/18 hr)	IgE (um/ml)	Eosinophils (cells/cu mm)	Clinical score
G. F.	24	F	104	80	170	29.0
M. M.	19	M	211	1800	921	27.0
R. S.[2]	19	M	41	300	544	29.0
G. W.	14	M	95	98	125	26.0
M. R.	13	F	88	N.D.	200	24.0
B. A.	13	M	151	1200	762	25.0
D. T.	13	M	299	2250	391	22.0
D. B.	12	M	97	3600	1743	17.0
S. H.	12	M	181	2800	501	12.0
T. B.[2]	12	M	39	1200	966	24.0
T. C.[2]	12	M	54	1500	564	25.0
R. C.	10	M	127	8800	131	23.0
D. S.	9	M	62	1980	210	5.0
B. L.	8	M	49	2400	3158	4.0
G. T.	8	M	161	120	83	27.0
W. H.	6	F	228	1600	514	18.0
D. H.	5	F	14	110	1103	14.0
S. B.	4	F	25	230	2875	22.0
M. M.	3	M	166	4000	1733	12.0
D. M.	2	M	12	142	797	25.0
K. M.	2	F	89	480	464	29.0
N. A.	1.5	M	7.0	21	249	29.0

[1] Normal age-matched chemotaxis values (mean ± 1 S.D.): 215 ± 72 (11+ years); 142 ± 64 (6 to 10 years); 71 ± 55 (3 to 5 years); 31 ± 25 (0.1 to 2 years).

[2] Monocyte values >2 S.D. below normal.

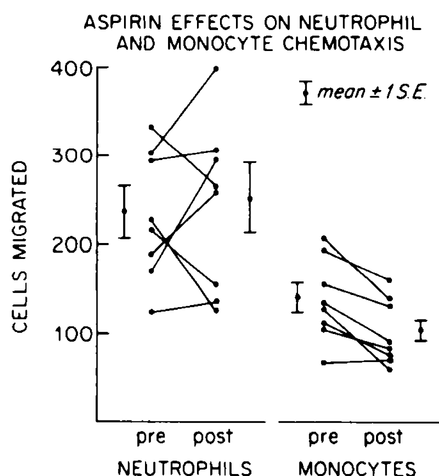

Fig. 3. Neutrophil and monocyte chemotaxis pre- and postaspirin ingestion in normal adults. *Brackets*, mean ± 1 S.E. Predose mean neutrophil value (232) is not significantly different from post mean value (249); predose monocyte value (137) is significantly different from postmean value (100), $P < 0.05$.

hydrocortisone were generally in excess of plasma cortisol levels following IV administration in status asthmaticus (100 μg/100 ml) (5). The inhibiting concentrations of ephedrine and diphenhydramine also exceeded observed plasma concentrations following PO administration (2, 18). Aspirin levels of 20 and 50 ml/dl produced significant inhibition of chemotaxis; however, these same levels caused significant loss of cell viability at 18 hr as measured by trypan blue exclusion (73 and 53% viability, respectively.) For the other tested drugs, the percentage of mononuclear cell viability exceeded 90% for all drug centrations except diphenhydramine (250 μg/ml, 84% at 3 hr, 88% at 18 hr; 1000 μg/ml, 59% at 18 hr, 250 μg/ml, 45% at 18 hr), and hydrocortisone (100 μg/ml, 86% and 250 μg/ml, 83%).

In vivo aspirin studies are shown in Figure 3. Aspirin levels ranged between 18 and 29 mg/dl with a mean of 22.4 mg/dl. A predose mean neutrophil chemotaxis value of 232 migrated cells was not significantly different from a postmean value of 249. When monocyte chemotaxis was measured concurrently, a uniform decrease in 7 of 8 subjects was observed. A preaspirin mean value of 137 cells significantly differed from a postaspirin value of 100 cells.

DISCUSSION

Increased interest has developed concerning the role of monocyte chemotaxis in normal host defense and possible aberrations of this function in disease states or secondary to pharmacologic agents. The agarose technique provides a simple and reproducible method to study chemotaxis and allows confirmation and extension of data previously obtained by the Boyden method (6, 15, 21).

Defective monocyte chemotaxis has previously been described in severe chronic mucocutaneous candidiasis (11, 27). Our patient resembles Gallin's patient who, in addition to mucocutaneous candidiasis, has recurrent pyogenic infections, defective delayed hypersensitivity, and lack of response to transfer factor. In addition, *in vitro* monocyte killing of *C. albicans* has been consistently impaired in our patient and may contribute to his increased clinical severity. Combination defects of monocytes involving two separate functions, namely directed movement and killing, have a parallel in combined polymorphonuclear defects (20).

Investigations of monocyte function in patients with atopic dermatitis are a logical extension of previous studies noting abnormalities of cell-mediated immunity in this disease (13, 19, 23). Immunodeficient states associated with eczema, *i.e.*, Wiskott-Aldrich syndrome, also have defects in monocyte chemotaxis, apparently due to increased levels of circulating lymphocyte-derived chemotactic factor (1).

Rogge and Hanifin (25), using a Boyden chamber method, found decreased polymorphonuclear chemotaxis in seven of 11 adult atopic patients and defective monocyte chemotaxis in four of four patients. Impairment of cell movement correlated with clinical severity but not with total serum IgE. Subsequent studies (14) showed inhibition of PMN chemotaxis with sera from three patients. During clinical remissions, these sera did not cause inhibition. In another study, Furakawa and Altman (10), using Boyden chambers, noted depressed monocyte chemotaxis in 8 of 17 atopic children with hyper-IgE (>1000 IU/ml) and two of nine nonhyper-IgE atopic children. No cell-directed chemotactic inhibition was found in the plasma of these patients. Snyderman *et al.* (29) found inhibition of monocyte chemotaxis in 8 of 14 atopic dermatitis patients. Of these 8 patients, sera from 5 of 6 tested demonstrated inhibition of chemotaxis. Depressed monocyte chemotaxis did not correlate with serum IgE concentrations, severity of eczema, or the presence of infection. Furthermore, *in vitro* incubation of normal monocytes with histamine or IgE myeloma did not cause inhibition.

Our study using an agarose method showed a lower percentage of patients (3 of 22) with monocyte depression. These monocyte values did not correlate with serum IgE levels, blood eosinophils, or clinical severity. No serum inhibitors were demonstrated.

Multiple reasons may account for the differences among studies in the percentage of monocyte chemotaxis defects observed. First, chemotaxis methodologies may selectively enhance or suppress cell movement. Gallin *et al.* (12), for example, noted abnormal random monocyte chemotaxis in patients with Chediak-Higashi syndrome when a Boyden chamber was used. However, with a capillary tube system (which, like the agarose system, does not use a filter), normal chemotaxis was observed. Other major differences in chemotaxis methodologies include choice of chemoattractants (lymphocyte-derived chemotactic factor in Snyderman's study; zymosam-activated serum and bacterial chemotactic factor by Furakawa *et al.*; zymosan-activated serum in our study) and methods to study serum inhibitors (preincubation and wash in our studies and the studies of Furakawa *et al.*, direct addition of 10% serum to cell preparations by Snyderman *et al.*).

A second major area causing differences among these three studies is patient and control selection. As shown by our previous study (15), monocyte chemotaxis is age-dependent with values increasing with age. In the study of Snyderman *et al.*, 5 of 8 patients with "depressed" monocyte chemotaxis are under 5 years of age but are compared to 98 normal adult subjects and 5 disease-control children with recurrent infections. If only adult values were used in our study, an additional 6 patients would have "depressed" monocyte chemotaxis.

Patient selection is also hampered in all studies by the inability to clinically define with accuracy the presence of infections and the extent and severity of skin involvement. As shown by Leyden *et al.* (17) atopic patients apparently "free" of skin infection have increased numbers of *Staphylococcus aureus* in clinically normal skin. Staphylococcal products also demonstrate a complex pattern *in vitro* of either stimulation or inhibition of monocyte and neutrophil chemotaxis (26).

Finally, drug administration to atopic patients may influence chemotactic results. Our *in vitro* data showed suppression of monocyte chemotaxis by theophylline in the therapeutic range. These *in vitro* studies do not take into account the effect of chronic administration or the action of drug metabolites, protein binding, and other factors operative *in vivo*. However, they confirm previous studies of cell movement which showed a decreased chemotactic response to agents which increase cyclic AMP (4, 9, 24, 30).

Our observation of greater suppression of monocyte than of neutrophil chemotaxis with PO administered aspirin parallels data from animal studies and suggests selective inhibition of chemotaxis depending on cell type. In rabbits, Borel and Feurer (3) noted a marked *in vitro* inhibition of macrophage chemotaxis with phenylbutazone and sodium salicylate but little effect on PMN chem-

otaxis. DiRosa *et al.* (7) using a rat-paw model, showed that anti-inflammatory drugs cause a suppression of mononuclear cells but not polymorphs.

Further, *in vivo* and *in vitro* studies on the effects of aspirin on human monocyte function are needed to better elucidate the mechanism of action of this widely used anti-inflammatory agent.

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