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A BIOCHEMICAL STUDY OF THE COTTON PELLET GRANULOMA IN THE RAT

EFFECTS OF DEXAMETHASONE AND INDOMETHACIN

PHILIP J. BAILEY,* ANITA STURM and BASILIO LOPEZ-RAMOS
Department of Immunopharmacology, Merck Institute for Therapeutic Research, Rahway, NJ 07065, U.S.A.

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Abstract—The subcutaneous implantation of a cotton pellet into a rat results in the formation of a granuloma at the site of the implant. The early events comprise an accumulation of fluid and proteinaceous material together with an infiltration of neutrophils. The granuloma formed by day 7 is characterized by the formation of a vascularized fibrous capsule containing fibroblasts and infiltrating mononuclear cells which are rich in *N*-acetyl-β-D-glucosaminidase (NAG). Granuloma development was quantitated by dry weight measurements, and its cellular content was measured by assaying activity of NAG and total nucleic acid content. Nucleic acid determinations showed that cell infiltration into the granuloma took place at a virtually constant rate over a 7-day period. In contrast, the NAG activity did not change significantly until after day 5 when a large increase in the amount of enzyme extractable from the granuloma was seen. Systemic treatment of the animal with dexamethasone or indomethacin resulted in an inhibition of granuloma weight gain, NAG activity and nucleic acid levels. The data suggest that the two drugs acted during the early phase of granuloma development at the level of cell infiltration. Both drugs given on days 0–3 alone suppressed granuloma formation, whereas treatment on days 4–7 was without effect.

The classical assay of chronic granulomatous responses resulting from the implantation of a pellet of cotton wool measures the increase in dry weight of the implanted pellet [1] and, thus, includes fluid phase extracellular components such as ground substance, salts and extracellular proteins in the overall dry weight. Relatively little attention has been given to the cell populations that infiltrate such lesions. Despite its imprecision and low sensitivity, this assay has proven useful for the demonstration of pharmacological activity of steroids as anti-inflammatory agents [2, 3]. On the other hand, the sensitivity of the method to non-steroidal anti-inflammatory agents is often very poor.

By using biochemical markers, information can be obtained about cell-related events that occur in the granuloma, which greatly enhances the sensitivity of the system, particularly in relation to the effects of the non-steroidal anti-inflammatory drugs. Measurement of the total nucleic acid content together with a representative lysosomal enzyme, *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30, NAG) which is present in high specific activity particularly in macrophages, has enabled us to monitor certain aspects of cellular infiltration and localization within the implanted pellet. The time course of these variables in the cotton pellet granuloma has been measured together with the effects of dexamethasone and indomethacin as representatives of steroidal and non-steroidal anti-inflammatory drugs respectively. The data suggest that these two drugs act during the early phase of granuloma development at the level of cell infiltration.

* Address reprint requests to: Philip J. Bailey, Ph.D., Dept. of Immunology, Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, NJ 07065, U.S.A.

EXPERIMENTAL PROCEDURE

Implantation of cotton pellets. Cotton pellets weighing 41–55 mg were cut from dental rolls (Johnson and Johnson No. 1–6″) and were sterilized by autoclaving. Prior to implantation each pellet was treated with 0.2 ml of antibiotic solution (10,000 units/ml penicillin and 10,000 μg/ml streptomycin). Cotton pellets were implanted into male Sprague–Dawley rats (200–250 g, Taconic Farms, Germantown, NY) under Metofane (methoxyflurane, Pitman-Moore, Inc., Washington Crossing, NJ) anaesthesia. A small ventral, mid-line incision was made, and the dermis was separated from the underlying peritoneal wall by insertion of a blunt-ended pair of scissors. Two pellets were implanted in each rat, one on each side of the incision. The incision was closed with a stainless steel clip.

Sampling and extraction of granulomas. Rats were killed by asphyxiation in carbon dioxide. Cotton pellets and the accompanying granulomatous tissue were removed from the rats with a pair of forceps by reflecting the overlying skin and removing the granuloma together with a small amount of any capsular material present. One granuloma was usually placed in a glass Petri dish, air dried at 60° for 18 hr, and weighed. The other granuloma was used for biochemical assays and was frozen in dry ice and kept frozen at −10° until processed.

The frozen granuloma was extracted using a technique developed by Dr. Charles Gitterman and Mrs. Silvi Luell of these laboratories. The granuloma was placed in 9.0 ml of ice-cold 0.9% saline containing 0.1% (v/v) Triton X-100. The granuloma was then subjected to disintegration in the Polytron disintegrator (Polytron, Kinematica GMBH, Luzern, Switzerland) for 20 sec at maximum speed. The cotton and other solid material were pelleted by centrifugation at 1500 *g* for 10 min, with two centrifugations usually necessary to remove all the cotton. NAG and nucleic acid determinations were performed directly on the supernatant fractions. If the assays were not done immediately, the supernatant fractions were kept frozen until used.

Determination of N-acetyl-glucosaminidase (NAG) activity and nucleic acid levels. NAG activity was based on the hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in a modification of the procedure of Woollen *et al.* [4], adapted for the Technicon Autoanalyzer. Briefly, the sample aliquot (6.4 μl to 100 μl) is picked up by the probe and mixed in a three-way cactus with a stream of substrate (2.24 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide), and simultaneously percolated with a stream of air. The mixture of substrate, sample and air is thoroughly mixed by passing it along a coil of fourteen turns. The mixture enters the heating bath at 37° where it is incubated for 15 min. At the exit from the heating bath the stream is joined by a stream of 0.2 M glycine buffer (pH 10.4) which stops the reaction. The mixture proceeds to the flow cell of the spectrophotometer where the *p*-nitrophenol liberated by the action of the enzyme on the substrate is measured at 400 nM. A tracing proportional to the amount of *p*-nitrophenol is described on the recorder. From the height of the tracing and extinction coefficient of the *p*-nitrophenol under the actual conditions of the experiment, enzyme activity is calculated and expressed as μmoles *p*-nitrophenol liberated per hour.

The nucleic acid content of the granuloma was determined fluorometrically in the autoanalyzer. The method is specific for DNA and RNA and is based on the original ethidium bromide reaction developed by Le Pecq *et al.* [5]. Equal amounts of polytron supernatant fluid and ethidium bromide solution (10–20 μg/ml) in calcium-free Krebs buffer (pH 7.4) are mixed in a three-way cactus. The stream of ethidium bromide and sample is segmented by a stream of air and incubated at room temperature by passage through a mixing coil of twenty-eight turns (0.2 mm diameter). The outflow from the mixing coil goes directly to the flow cell of a Turner or Aminco spectrofluorimeter and the relative fluorescence is measured. A series of DNA standards are subjected to the same procedure, and a standard curve is obtained. From the slope of the standard curve (which is linear past 100 μg/ml) and the value of the relative fluorescence (obtained by the height of the tracing), the amount of DNA and RNA is determined and expressed as μg total nucleic acid/per milliliter. An interesting finding was that the nucleic acid could be extracted from the infiltrated pellet just by treatment with the Polytron for 20 sec at top speed in saline/Triton solution. That the measurements were indeed specific for DNA and RNA was proved by removing all fluorescence by addition of DNase and RNase.

Control experiments showed that the cotton prior to implantation contained no measurable nucleic acid or NAG. The absolute values found for the NAG and nucleic acid contents of the granuloma varied from one experiment to another. Since homogenization in saline/Triton solution extracts NAG and nucleic acid, both these variables were measured in the same extract and reported as paired values in Results.

Administration of drugs. Drugs were administered to rats by subcutaneous injection in a 0.5 ml volume in the nape of the neck. Both dexamethasone and indomethacin were suspended in a vehicle of 0.9% NaCl, 0.5% carboxymethylcellulose, 0.4% Tween 80 and 0.9% benzyl alcohol. A fine suspension of drug was obtained by sonication of the drug suspension in a MSE ultrasonic power unit (Instrumentation Associates Inc., New York, NY). Drug dosages were selected which gave maximum or near maximum effects on the NAG, nucleic acid and dry weight.

RESULTS

Kinetics of enzyme, nucleic acid, and weight gain in the developing cotton pellet granuloma. Figure 1 shows the development of the granuloma over a period of 10 days as indicated by dry weight, NAG activity and nucleic acid levels. Determinations of dry weight gain and NAG activity indicated that there was a lag period of 5 days during which time there was little change in these two variables. Between days 5 and 7, however, there was a rapid change with a 3-fold increase in NAG levels during this 48-hr period together with a somewhat smaller gain in dry weight during the same period. The

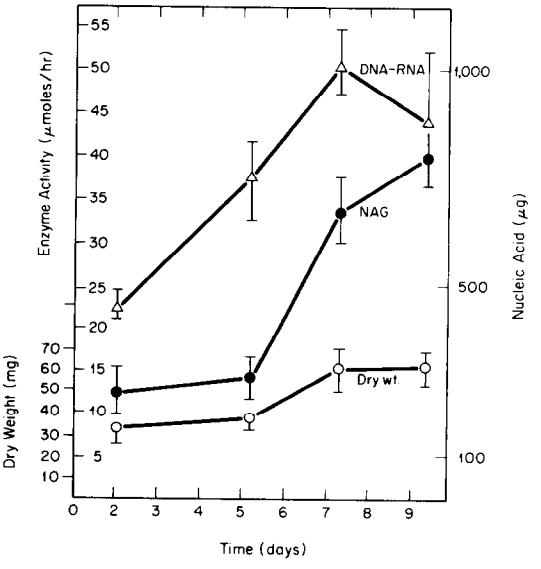

Fig. 1. Changes in *N*-acetyl-β-D-glucosaminidase (●) activity, increase in granuloma dry weight (○), and total extractable nucleic acid (△), over a period of 10 days *in vivo*.

Table 1. Effects of dexamethasone and indomethacin on dry weight and NAG activity of the 7-day cotton pellet granuloma

Treatment	Increase in dry wt* (mg)	Significance	NAG* (μmoles/hr)	Significance
Vehicle	45.0 ± 11.9		31.9 ± 8.3	
Dexamethasone ($75\ \mu g \cdot kg^{-1} \cdot day^{-1}$)	18.0 ± 1.9 (60%)	$P < 0.01$	6.2 ± 2.7 (80%)	$P < 0.001$
Indomethacin ($3.0\ mg \cdot kg^{-1} \cdot day^{-1}$)	29.0 ± 9.3 (36%)	$P < 0.05$	14.1 ± 1.6 (56%)	$P < 0.001$

* Results are expressed as means ± S.D. Percent inhibition is shown in parentheses.

increases in both NAG activity and dry weight were sustained throughout the entire 10 days of the experiment. Nucleic acid determinations, which reflect total cell infiltration into the cotton, showed a steady gain throughout the experiment, which was in agreement with histologic findings. The data indicate that there was a continued accumulation of cells in the granuloma during the first 5 days "lag period" when enzyme and dry weight were not changing significantly. It is noteworthy that the rate of increase in NAG activity with time was greater than that of weight gain, suggesting that measurement of NAG activity was a more sensitive variable when determining the effect of drugs on the development of the granulomas.

Effects of dexamethasone and indomethacin on dry weight and NAG activity of the cotton pellet granuloma. Cotton pellets were implanted into groups of six rats which were then dosed with either 75 μg/kg dexamethasone, 3 mg/kg indomethacin, or aqueous vehicle, each group receiving one subcutaneous injection daily. On day 7 after implantation, the granulomas were removed from the animal, and extracts were made. The data in Table 1 show that both dexamethasone and indomethacin inhibited increases in pellet dry weight and NAG activity of the granuloma. NAG activity was much more sensitive to the effects of drug, however, than was the dry weight measurement, the enzyme showing greater inhibition than the dry weight at the same dose level. This was particularly noticeable with indomethacin, which only affected dry weight to a small extent.

Effects of dexamethasone and indomethacin on the kinetics of the development of the cotton pellet granuloma. The data shown in Figs. 2 and 3 show that NAG and nucleic acid levels of the granuloma were affected by treatment with dexamethasone and indomethacin. Dexamethasone was the more effective compound in this assay, producing a larger significant effect at a lower dose level. Even as early as day 5 the effect of dexamethasone showed a statistically significant lowering of the NAG levels and dry weights of the granulomas, whereas on day 5 the effects seen with indomethacin were not statistically significant. Figure 3 shows that dosing with dexamethasone and indomethacin lowered the rate at which nucleic acid (and therefore cells) accumulated within the granuloma.

Effects of different dose schedules for dexamethasone and indomethacin on the development of the cotton pellet granuloma in the rat. In view of the 5-

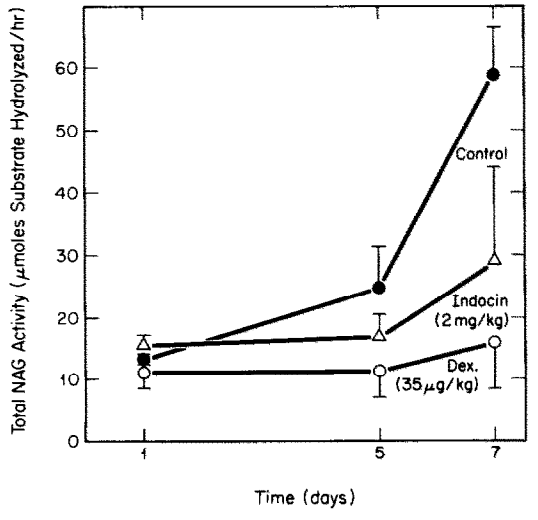

Fig. 2. Changes in total *N*-acetyl-β-D-glucosaminidase activity after dosing with aqueous vehicle (●), indomethacin 2 mg/kg (△), or dexamethasone 35 μg/kg (○). Animals received one daily dose administered subcutaneously.

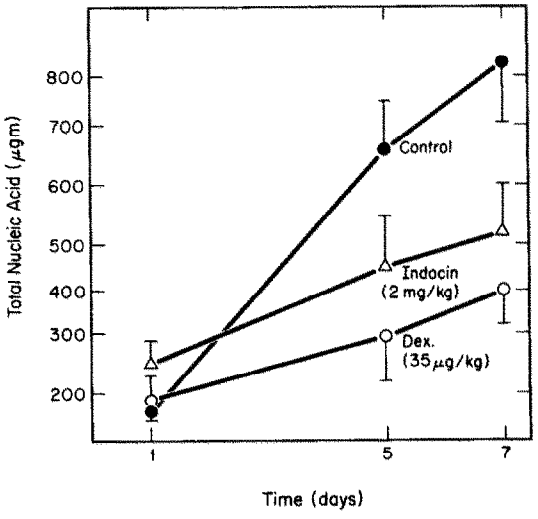

Fig. 3. Changes in the total extractable nucleic acid after dosing with aqueous vehicle (●), indomethacin 2 mg/kg (△), or dexamethasone 35 μg/kg (○). Animals received one dose daily administered subcutaneously.

Table 2. Effects of daily dosing on days 0–3 with dexamethasone or indomethacin on development of cotton pellet granulomas in rats*

	Increase in dry wt (mg)			NAG (μmoles/hr)			Nucleic acid (μg/pellet)		
Day	Control	Dexamethasone	Indomethacin	Control	Dexamethasone	Indomethacin	Control	Dexamethasone	Indomethacin
2	33.2 ± 6.2	29.9 ± 2.0 (10%)	31.9 ± 5.1 (4%)	12.6 ± 3	8.2 ± 2.3 (35%)	8.9 ± 2.8 (29%)	450 ± 47	450 ± 115 (0%)	347 ± 63 (23%)
5	39.5 ± 3.5	29.6 ± 3.2 (25%)	35.2 ± 3.8 (11%)	14.7 ± 3.1	6.6 ± 1.6 (55%)	10.0 ± 2.3 (32%)	756 ± 86	274 ± 106 (64%)	546 ± 136 (28%)
7	61.9 ± 11.0	31.0 ± 7.7 (50%)	56.3 ± 15.4 (9%)	34.4 ± 4.1	7.9 ± 1.6 (77%)	15.0 ± 2.4 (56%)	1006 ± 79	268 ± 77 73%)	668 ± 115 (34%)

* Results are expressed as means ± S.D. Percent inhibition is shown in parentheses.

Table 3. Effects of daily dosing on days 4–7 with dexamethasone and indomethacin on development of cotton pellet granulomas in rats*

	Increase in dry wt (mg)			NAG (μmoles/hr)			Nucleic acid (μg/pellet)		
Day	Control	Dexamethasone	Indomethacin	Control	Dexamethasone	Indomethacin	Control	Dexamethasone	Indomethacin
1	28.3 ± 8.7	27.7 ± 2.1 (2%)	27.7 ± 1.3 (2%)	11.1 ± 1.1	10.6 ± 2.6 (5%)	9.8 ± 1.4 (12%)	221 ± 44	222 ± 14 (0%)	222 ± 632 (0%)
5	34.3 ± 6.5	35.5 ± 4.5 (−4%)	35.6 ± 3.3 (−4%)	11.0 ± 2.2	10.13 ± 1.7 (8%)	12.6 ± 2.2 (−14%)	446 ± 47	484 ± 128 (−8%)	484 ± 128 (−8%)
7	45.3 ± 10.8	45.6 ± 5.8 (0%)	48.3 ± 9.4 (−7%)	28.1 ± 7.8	25.7 ± 8.8 (9%)	34.4 ± 6.9 (−22%)	626 ± 224	631 ± 218 (−1%)	734 ± 219 (−17%)

* Results are expressed as means ± S.D. Percent inhibition is shown in parentheses.

day lag period before the appearance of large amounts of NAG within the granuloma, it was of interest to determine the effect of drug treatment during the first 3 days of the granuloma development. This was compared with the effect of drug treatment during days 4–7, i.e. after the early events of the lag phase had already taken place. In the first experiment, rats implanted with two cotton pellets were treated with either dexamethasone (75 μg/kg, s.c.) or indomethacin (3.0 mg/kg, s.c.), dosing once daily on days 0–3. The NAG content of the pellet and the dry weight and nucleic acid increases were then measured on days 2, 5, and 7. The results are shown in Table 2. When animals were dosed only on days 0–3, the dry weight gain of the pellets on day 7 was suppressed 50% by treatment of the animals with dexamethasone, whereas the dry weight gain was not affected in animals treated with indomethacin under the same conditions. On the other hand, in the same animals NAG activity was affected significantly at day 7 by treatment with both dexamethasone and indomethacin, the effect on NAG levels of dexamethasone treatment on days 0–3 being considerably greater than that of indomethacin. Measurements of nucleic acid levels in the pellet suggested that under this regimen both drugs suppressed the influx of cellular material into the cotton rather than interfering with the synthesis of enzyme by cells already resident in the granuloma.

When animals were dosed on days 4–7 (Table 3), i.e. after the granuloma had time to establish and including the critical "activation" stages of days 5–7, the effect of the drugs was very different. Neither dexamethasone nor indomethacin had any effect on dry weight, NAG or nucleic acid levels.

DISCUSSION

This study has utilized the lysosomal enzyme marker *N*-acetyl-β-glucosaminidase (NAG) and nucleic acid levels to quantitate the cellular events in granuloma development and has compared this with the dry weight values as measured in the classical model of the cotton pellet granuloma. The use of NAG as a quantitative estimate of the intensity of cellular infiltration in response to chronic inflammatory stimuli (zymosan and asbestos) has been described by Schorlemmer *et al.* [6].

The kinetics of development of the granuloma in terms of cell accumulation proved to be very interesting. Very little change in the dry weight of the granuloma or the NAG activity (which remained at very low levels) could be demonstrated over the first 5 days. In contrast, nucleic acid determinations indicated that a very active cell accumulation was taking place during this period. Serial histologic sections through a control granuloma indeed showed an increasing number of PMNs penetrating the cotton from the periphery, many of which, from the pyknotic appearance of their nuclei, were dead cells. PMNs contain very low levels of NAG (unpublished results) and, therefore, the very active infiltration of PMNs during the first 5 days is not reflected in the NAG or the dry weight measurements.

Between days 5 and 7 there was a dramatic increase in the NAG activity which was paralleled, but to a smaller degree, by an increase in weight. Histologic studies showed that extensive vascularization was taking place with the formation of a fibrous capsule and the laying down of ground substance, together with a massive infiltration of the capsule by mononuclear cells, which contain NAG of high specific activity.

Measurements of granuloma nucleic acid levels suggest that both dexamethasone and, to a lesser extent, indomethacin were acting to suppress cell influx into the granuloma. It was shown histologically, for example, that dexamethasone inhibited virtually completely the infiltration by cells and the formation of capsule, whereas indomethacin treatment permitted virtually normal capsule development to take place. There were, however, fewer mononuclear cells infiltrating the granuloma after indomethacin treatment, and indeed some of the mononuclear cells could be seen to have lysed.

The data showed that dosing with drugs on days 0–3 ("the lag phase") was effective in inhibiting the development of the granuloma as measured by NAG determinations at day 7. This was surprising because the cell infiltration on days 0–3, measured by increased nucleic acid levels, was primarily by neutrophils, whereas the cell infiltration at day 7 was mainly by mononuclear cells. This suggests that inhibition of neutrophil accumulation in the early phases of granuloma development had an influence on the degree of mononuclear cell infiltration on day 7.

There are several possible explanations for this pattern of activity. Early events occurring in the vicinity of the cotton pellet result in the generation of substances chemotactic towards neutrophils and monocytes. Possibly products of the complement system such as the recently described complement derived chemotactic factors described by Orr *et al.* [7], the kinin system [8], or the generation of products of arachidonic acid oxidation [9] could all serve as candidates for chemotactic mediation.

The role of arachidonic acid oxidation products as possible stimulators of chemotaxis is particularly interesting in the present context. Non-steroidal anti-inflammatory drugs such as indomethacin inhibit the biosynthesis of prostaglandins [10] which is generally taken as the explanation for the anti-inflammatory action of the compounds. At low concentrations indomethacin is an inhibitor of the cyclooxygenase pathway of arachidonic acid oxidation without significantly affecting the lipoxygenase pathway [11]. The lipoxygenase pathway of arachidonic acid oxidation, however, results in the generation of hydroxy acids which are chemotactic, at least for granulocytes [12]. Indomethacin at the doses used in this study would not be expected to affect significantly the lipoxygenase pathway, which might account for the relatively weak effect of indomethacin compared with dexamethasone on cell infiltration into the granuloma. In contrast, Walker *et al.* [13] showed that prostaglandin synthesis and leukocyte migration are independent phenomena.

Dexamethasone, on the other hand, has been shown to hinder the release of arachidonic acid from biological membranes and would, therefore, be expected to inhibit the production of intermediates

of both the lipoxygenase and the cyclooxygenase pathway of arachidonic acid oxidation by decreasing the availability of free arachidonic acid. Dexamethasone treatment would therefore inhibit generation of prostaglandins of the E-type as well as the more potent chemotactic hydroxy acids generated by the action of lipoxygenase. This would then account for the more dramatic effect of dexamethasone on cell infiltration as compared with the much weaker effect of indomethacin. The question as to whether arachidonic acid oxidation products are stimuli for cell infiltration in the cotton pellet granuloma, however, is still an open one.

It is possible that the mediators causing cell infiltration in cotton pellet implants are not products of arachidonic acid oxidation but are related to breakdown products of the complement pathway. A great deal of evidence suggests [14] that the complement system, through cleavage of its fifth component to form C5a, is a major source of chemotactic activity, and indeed during the early phases of granuloma formation it may be that complement derived chemotactic products are the major factors attracting cells into the lesion. The anti-inflammatory effects of dexamethasone and indomethacin are possibly mediated through inhibition of the formation of these chemotactic mediators or by suppression of the ability of inflammatory cells to respond to a chemotactic stimulus. We have no evidence at the present time to support such a mechanism of action.

The main conclusion to be drawn from this study is that both dexamethasone and indomethacin inhibit the development of cotton pellet-induced granulomas in the rat by suppressing the early infiltration of neutrophils into the granuloma. Cell-associated variables, such as synthesis of NAG, are a more sensitive measure of drug activity than is the classical dry weight determination. The cotton pellet granuloma assay when used in conjunction with the enzyme assays enables potential anti-inflammatory compounds to be studied from the point of view of cell recruitment and also cell activation to produce potential mediators of inflammation such as lysosomal enzymes. Use of the cotton pellet granuloma test in this manner expands the potential of the assay to encompass new anti-inflammatory compounds with novel modes of action.

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