EXPERIENCES IN THE SEARCH FOR ANTI-INFLAMMATORY AGENTS OF MICROBIAL ORIGIN

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Whole shaken cultures of 20 random, unidentified actinomycetes were extracted with *n*-butanol at pH 4.5, 7.0, and 8.5, respectively. Residues of butanol-extractable materials (BXM) were reconstituted (100×) in buffers and freeze-dried. BXM were surprisingly well tolerated in animals and were screened against influenza A viral pneumonia in mice. One culture yielded BXM-80 which suppressed both chemical (LPS) and viral (NDV) pneumonia in mice as well as inhibited rat foot pad edema induced by carrageenin.

Aspirin, Butazolidin, hydrocortisone, indomethacin, and prednisolone, which are known to inhibit carrageenin-induced rat foot pad edema were tested against chemical (LPS) and viral (NDV) pneumonia in mice. Only hydrocortisone and prednisolone suppressed LPS pneumonia. All of these 5 compounds failed to inhibit NDV pneumonia. Microbial products are suggested as a source for new and unique anti-inflammatory agents.

Utilization of microbial products as potential sources of unique pharmacodynamic agents has received increasing attention. The anti-inflammatory bacterial product, xerosin[1], was first detected in an antiviral screen using mice infected with influenza A virus[2]. Further studies showed that xerosin inhibited the non-transmissible pneumonia produced in mice by Newcastle disease virus (NDV)[3] and the chemical pneumonia produced by endotoxin (LPS)[4]. Later, xerosin was shown to have a wide variety of typical anti-inflammatory properties[5~7] but differed from cortisone in that xerosin suppressed viral pneumonia while cortisone failed to do so[4]. Xerosin was found to be heat-stable, non-dialyzable and insoluble in common organic solvents[1,2]. Extensive sophisticated efforts to obtain suitable purified preparations for further development were unsuccessful. In the studies described below, a systematic search for butanol-soluble anti-inflammatory microbial products was undertaken. Twenty unidentified antinomycetes were isolated from various soils and butanol-extractable materials (BXM) were obtained from shaken cultures. The BXM were screened in mice previously infected with influenza A virus. The results show that at least one of these 20 cultures yielded anti-inflammatory preparations of interest.

Materials and Methods

Actinomycetes, Fermentation and Extraction.

During an automobile trip from Florida to Alaska, 94 samples were collected from a wide variety of soils. Samples were taken at least 200 miles apart, placed in sterile 5-ml screw cap vials and stored at −10°C in the laboratory. From these samples, 20 apparently different actinomycetes were isolated using chitin agar medium and were maintained on EMERSON agar slants. Pre-inoculum shaken cultures [50 ml/250 ml (Erlenmeyer flask)] were seeded with suspended growth from an agar slant into M-5 medium (5 g dextrose, 5 g peptone, 5 g yeast extract, and 5 g beef extract in 1 liter of tap water) and

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incubated for 96 hours at room temperature on a rotary shaker at 150 rpm. Inocula were prepared by seeding 10 ml of the pre-inoculum into each of 18 flasks containing 100 ml of Hy Soy medium (7.5 g Hy-Soy-T-Sheffield, 20 g dextrose, 5 g NaCl and 2 g yeast extract in 1 liter tap water at pH 6.5). After 72 hours of incubation, 10 ml of the inoculum was seeded into 180 flasks and incubated 3~5 days (luxuriant growth) as above. The 180 shaken cultures were pooled, divided into 3 aliquots, and stored in plastic containers at −10°C.

The frozen candidate pooled cultures were thawed and serially thrice extracted with *n*-butanol (final: 20% v/v) for 30 minutes with agitation (rotary shaker) at pH 4.5, 7.0 and 8.5 respectively. The contents were transferred to appropriate separatory globes and allowed to separate overnight. The aqueous phases were discarded and the solvent-mycelial emulsions were centrifuged at 2,000 rpm for 10 minutes. The butanol layer was aspirated and evaporated under vacuum at 45°C to dryness. The residues were reconstituted in an appropriate aqueous solution (0.01 N Na_2CO_3, 0.01 N H_2SO_4, or distilled H_2O) at a concentration of about 50 mg/ml. This solution was distributed in 1.0-ml aliquots among 5-ml vials which were then freeze-dried under vacuum and stoppered in dry nitrogen. The preparations were stored at −10°C.

Animal Studies.

The procedures used have been previously described[2~4]. Male, Swiss-Webster ICR, 20~25 g (Southern Animal Farms, Prattville, AL) were used. LPS: *Escherichia coli* (lipopolysaccharide B) Difco, was diluted to 100 mg/0.5 ml in 0.9% saline and frozen in 4-ml aliquots at −20°C. Newcastle disease virus, (NDV), California strain, was passed in eggs and frozen at −70°C in 5-ml aliquots as undiluted allantoic fluid containing $10^{9.1}$ EID_{50} per ml.

Frozen inoculum pools of influenza A virus (IAV), mouse adapted PR-8 strain, consisted of 10% mouse lung suspensions containing $10^{8.7}$ ID_{50} per ml I. N. for mice. Appropriate dosages were determined by I.N. instillation of serial dilutions of frozen stocks of NDV[8,9], IAV[10] and LPS[4], respectively, into groups of 10~20 mice each. The mice were killed 3 days post instillation, and the lesion scores and lung weights were recorded. Dosages of NDV(1/8), IAV (1/100) and LPS (20 μg), respectively, were selected that killed 20~30% of the mice and produced severe pneumonia in the survivors on the third day after instillation. Mice were lightly anesthesized, using a covered 2-liter beaker containing 5~10 ml of ether. The jar was gently warmed by a household heating pad, and the mice were separated from the ether by a wire platform covered with cotton. Each mouse was removed the instant complete relaxation occurred and 0.05 ml was slowly dispensed onto the nares using a Schwarz Mann, 012 Biopette with a 0.05 ml adaptor. Mice instilled with NDV, IAV, or LPS together with controls were injected twice daily I.P. with graded doses of each preparation and the lungs were scored and weighed on the third day after injection[4]. A difference in lung weight of >100 mg was considered as evidence of anti-inflammatory activity.

Aspirin, Butazolidin, hydrocortisone, indomethacin, prednisolone and xerosin[1] were appropriately diluted in 0.5% carboxymethyl-cellulose. Preliminary toxicity tests were done by injecting 0.2-ml amounts of 2-fold serial dilutions of candidate preparations twice daily I.P. for 3 days into groups of 10 mice each. The mice were observed for 7 days and symptoms and mortality and necropsy findings were recorded. The LD_0 was used as the highest dosage to be used in further tests. All experiments were terminated 3 days after instillation of LPS, NDV, and IAV when inflammation, manifested as pneumonia, was maximal. The lungs of each mouse were weighed individually and scored as previously described[4]. The mean lung weights, standard derivations, and standard errors of the various groups were calculated. The statistical significance of the differences between control and treated groups was determined, using the STUDENT T test.

Results

Table 1 summarizes pertinent data on butanol-extractable materials (BXM) obtained from each of 20 unidentified actinomycete cultures. These preparations represented a 100× concentration of BXM from whole shaken cultures harvested at maximal growth. Pooled, whole shaken cultures (7.5 liters)

Table 1. Summary of data from primary screen: Suppression of influenza A virus (IAV) pneumonia in mice by butanol-extractable materials from shaken cultures of unidentified actinomycetes

Culture		Butanol-extractable materials			Suppression IAV mouse pneumonia		
No.	pH	pH	Yield	LD_0	200	100	50
			mg/liter	mg/kg	mg/kg	mg/kg	mg/kg
29	4.7	4.5	336	50			*
		7.0	254	13			*
		8.5	272	50			*
30	5.5	4.5	192	>400		−	
		7.0	154	>400	−	−	
		8.5	256	>400	−		
31	7.3	4.5	201	>100		−	
		7.0	322	>100		−	
		8.5	322	>100		−	
33	5.9	4.5	795	>400			+
		7.0	828	>400	+	−	−
		8.5	440	>200	+	+	−
34	5.5	4.5	353	>200		−	
		7.0	268	>200	−	−	
		8.5	323	>200		−	−
36	4.9	4.5	117	>200		−	
		7.0	133	>200		−	
		8.5	174	>200		−	
37	5.0	4.5	228	>400	−	−	−
		7.0	196	>200	+	−	−
		8.5	138	>400	−	−	−
41	5.5	4.5	248	>200	−		
		7.0	365	>200	−		
		8.5	286	>200	−		
60	5.4	4.5	396	>400	−	−	+
		7.0	406	>400	−	+	−
		8.5	368	>400	−	−	−
61	6.7	4.5	174	>400		−	−
		7.0	234	>400	−	−	
		8.5	256	>400	−	−	
62	6.8	4.5	109	>400		−	
		7.0	124	>400		−	
		8.5	124	>400		−	
64	5.2	4.5	221	>200	−		
		7.0	261	>200		−**	
		8.5	231	>200		−**	−
68	6.2	4.5	393	>400	−	−	−
		7.0	369	>400	−	−	−
		8.5	330	>400	−		
70	5.0	4.5	384	200		−	
		7.0	323	100		−	
		8.5	251	100		−	
75	5.9	4.5	351	>400	−		
		7.0	288	>400		−	−
		8.5	319	>400	−	−	
77	7.2	4.5	138	>400			−
		7.0	140	>400	−	−	−
		8.5	144	>400	−		
80	7.0	4.5	160	>200		+	+
		7.0	280	>400	+	−	−
		8.5	304	>400	+	+	
84	4.9	4.5	302	>400	−	−	−
		7.0	240	>400	−	−	−
		8.5	206	>400	−	−	−
89	5.7	4.5	618	200		−	
		7.0	550	100		−	
		8.5	484	100			−

Table 1. (continued)

Culture		Butanol extractable materials			Suppression IAV mouse pneumonia		
No.	pH	pH	Yield	LD_0	200	100	50
91	6.5	4.5	350	13			—*
		7.0	288	13			—*
		8.5	268	13			—*
Uninoculated culture medium		4.5	< 50			—	
		7.0	< 50			—	
		8.5	< 50			—	

* Toxic preparation: Tested at 13 mg/kg.

** Presumptive positive (±): Average lung weight reduced by > 75 mg.

Table 2. Suppression of influenza A virus (IAV) pneumonia in mice by butanol-extractable materials (BXM) of actinomycete culture 80 (See Table 1)

IAV-I.N. instilled	BXM-80		Lung lesion scores	% L/M	Av. wt. lungs (mg)
	pH	mg/kg			
log 4.2	4.5	100	5, 4, 2, 1, 1, 1, 1, 0, 0.	33	330
		50	3, 2, 1, 1, 1, 1, 1, 0, 0.	22	313
	7.0	200	5, 4, 2, 2, 2, 1, 1, 1, 1.	42	335
		100	5, 3, 3, 2, 2, 1, 1, 1, 0.	40	361
		50	5, 4, 4, 3, 3, 3, 1, 1, 0.	53	376
	8.5	200	1, 1, 1, 1, 1, 1, 0, 0, 0	13	292
		100	5, 3, 2, 2, 1 1, 0, 0.	35	331
		50	5, 5, 4, 4, 3, 2, 2, 2, 1.	62	438
4.2	Diluent		5, 5, 5, 5, 5, 5, 5, 3, 3, 3, 2, 2, 2, 2, 2, 1, 1, 1.	63	438
Saline	Diluent		1, 0, 0, 0, 0, 0, 0, 0, 0, 0.	2	267
nil	nil		0, 0, 0, 0, 0.	0	234

BXM-80: Butanol-extractable material from shaken cultures extracted at pH 4.5, 7.0, and 8.5, respectively (see Materials and Methods).

Lung lesion scores: 5=dead mouse with consolidated lungs; 4=75~100% lung tissue consolidated; 3=50~74%; 2=25~49%; 1=10~24%.

% L/M: Total lesion score ÷ total maximum lesion score ×100

were divided into 3 aliquots and extracted at pH 4.5, 7.0, and 8.5, respectively. Acute toxicity tests were done on each preparation by injecting 400, 200, 100, and 50 mg/kg I.P. into groups of mice each and observing them for 5 days after injection. The data show that the yields of BXM ranged from 109 to 828 mg/liter averaging 293 mg/liter. These preparations were surprisingly well tolerated in mice receiving a single I.P. injection. Of the 60 preparations tested, 50 were well tolerated (LD_0) at > 200 mg/kg and only 2 were lethal at < 50 mg/kg. It will be seen that of 20 actinomycete cultures, BXM-33

and BXM-80 contained biologically active materials. Details of the data on BXM-80 are shown in Table 2 for illustrative purposes.

BXM-33 and BXM-80 failed to inhibit *Candida albicans*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella schottmuelleri*, or *Streptococcus pyogenes in vitro*. Each preparation was tested at levels of 1, 0.1 and 0.01 mg/ml. Whatman filter paper (no. 4) strips were immersed in test solutions, carefully drained and placed on nutrient agar plates. One hour later,

Table 3. Effects of selected anti-inflammatory agents on chemical (LPS) and viral (NDV) pneumonia in mice

Preparation		Reduction in mean lung weight					
		LPS			NDV		
		red. in lung wt.	P	M.E.D.	red. in lung wt.	P	M.E.D.
Aspirin	mg/kg	mg.		mg/kg	mg.		mg/kg
	200*	+49					neg.
	100	+30					
	50	2		neg.			
	25	+32			n.t.		
	12.5	+ 2					
Butazolidin	100*	+27		neg.	30		
	50	9			22		neg.
	25	+13			12		
	12.5	+ 8			19		
Hydrocortisone	200*	120	<0.01		6		
	100	104	<0.01		+46		
	50	103	<0.01	50	+43		neg.
	25	15			+28		
	12.5	53					
Indomethacin	3.0*	+27		neg.	56	0.05	neg.
	1.5	35			6		
	0.75	1			29		
	0.3	+ 9			7		
	0.15	+22					
	0.08	+10					
Prednisolone	200	99	<0.01				
	100	91	<0.01				
	50	97	<0.01	50	n.t.		
	25	57					
	12.5	+ 7					
	6.3	22					
Xerosin	100*	93	<0.01				
	50	108	<0.01	12.5	46	<0.02	
	25	83	<0.01		58	<0.01	25
	12.5	72	<0.01		47	<0.02	
	6.3	40	<0.1		45	<0.02	
	3.2				28		
BXM-80	100	91	<0.01		71	<0.01	
	50	74	<0.01		105	<0.01	
	25	74	<0.01	12.5	89	<0.01	<25
	12.5	83	<0.01				
	6.3	36					
	3.2	8					

Mean of individual lung weights from groups of 30~40 mice each.
LPS=lipopolysaccharide. NDV=Newcastle disease virus. M.E.D.=Minimal effective dose (2 daily injections). neg=negative at LD_0. n.t.=not tested. * LD_0.

perpendicular to the filter strip, 24-hour broth cultures were surface-streaked on the agar. Bacterial-inoculated plates were incubated at 37°C and those with yeasts at room temperature. The inoculated plates were examined at 20 hours to determine inhibitory effect.

Table 3 summarizes data from typical experiments, showing the effects of selected anti-inflammatory agents on LPS and NDV pneumonia in mice. It will be seen that hydrocortisone, prednisolone, xerosin and BXM-80 suppressed LPS pneumonia in mice, whereas aspirin, Butazolidin and indomethacin failed to do so. It will also be seen that xerosin and BXM-80 suppressed NDV pneumonia.

Arrangements were made with Dr. SOLOMON MARGOLIN (A.M.R. Inc., Princeton, N. J.) for standard pharmacological tests. BXM-80 was tested in rats and dosage levels of 200, 100, and 50 mg/kg against the foot pad edema produced by carrageenin[11] and compared with indomethacin at 3 mg/kg. Both BXM-80, at all 3 dosages, and indomethacin significantly ($P<0.01$) inhibited rat foot pad edema produced by carrageenin. Further, BXM-80 was found active (<50 mg/kg) in similar tests using adrenalectomized rats and to exhibit considerable analgesic activity at doses of 25, 50, and 100 mg/kg in edema induced in rats by silver nitrate although little anti-inflammatory effects were observed at these levels.

Cultures 33, 64 and 80 that yielded active preparations (Table 1) were found to do so reproducibly when fermentations and extractions were repeated. All of 36 consecutive replicate fermentations and extractions yielded preparations of BXM-80 of comparable potency (Table 3). Similarly, all of 7 and 5 consecutive preparations of BXM-33 and BXM-64, respectively, were found to reduce the average lung weights by >100 mg in mice instilled with LPS at dosage levels 50 mg/kg or less.

Preliminary developmental studies were done on BXM-80 using mean lung weight reduction of >100 mg (LWR) of LPS pneumonia as the bioassay. Whole culture, beer, and mycelium were each extracted with butanol in the usual manner (see Materials and Methods) and the results were as follows: Whole culture: yield = 433 mg/liter; LWR = 50 mg/kg. Beer: yield 111 mg/liter; LWR = 50 mg/kg; Mycelium: yield = 863 mg/liter; LWR = <50 mg/kg; indicating that mycelium was the best source of BXM-80. Filtration of aqueous solutions of BXM-80 through Whatman #4 paper and Nalgene 0.45 μ pads did not reduce its potency (LWR = 50 mg/kg) but removed more than 75% of the total solids. Further developmental studies are in progress and the results will be published elsewhere.

Discussion

The data presented here support the concept that microorganisms can provide unexpectedly rich sources of unique pharmacodynamic substances. Simple solvent extraction of 20 randomly isolated actinomycete cultures yielded preparations that were generally well tolerated in animals, at least one of which was pharmacologically active. BXM-80 shares many anti-inflammatory activities with the bacterial product, xerosin[1], but differs in its solubility in butanol, a property that should facilitate chemical purification. The potential utility of anti-inflammatory agents that also suppress viral pneumonia is obvious.

The applicability of viral (NDV) and chemical (LPS) pneumonia in mice as bioassays in evaluating anti-inflammatory agents has been described. Both IAV and NDV produce a typical interstitial viral pneumonia[8,9]. IAV pneumonia was selected for the primary screen because it represents a typical rapidly fatal viral infection in which the viral titer is maximal 18 hours after infection and declines during the development of pulmonary lesions[10]. On the other hand, NDV does not replicate in mouse lungs but produces pneumonia by the toxic effects of large numbers of NDV virions thus paralleling

many features of the natural disease in which viral titers are minimal or declining during active disease[8,9]. In contrast, chemical pneumonia produced in mice by instillation of endotoxin (LPS) was characterized by desquamation, atelectasis, lymphocytic infiltration and edema[4]. Hydrocortisone has been shown to suppress LPS pneumonia but not NDV pneumonia.

Previous studies have shown that daily injections of hydrocortisone[12] and xerosin[7,13] inhibited virus-induced ROUS sarcoma in chicks[14] resulting in small, hard, circumscribed tumors. Such tumors were obtained with hydrocortisone only when injections were begun before infection, whereas treatment with xerosin could be delayed until the day the tumors first appeared in the wing web. It will be recalled that when mycelial extracts of 55 random, unidentified actinomycete cultures were screened directly against virus induced ROUS sarcoma in chicks, 2 significantly and consistently delayed the latent period for tumor production[15].

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