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マウスの結核菌感染に対する Orotic acid ならびに 4-Amino-5-imidazolecarboxamide orotate の効果

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EFFECT OF OROTIC ACID AND 4-AMINO-5-IMIDAZOLECARBOXAMIDE OROTATE ON INFECTION OF MICE WITH TUBERCLE BACILLI*

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Since Schwartz et al[1]. reported that 6-mercaptopurine suppresses antibody formation, many papers appeared reporting on inhibition of immune response by purine analogues. In the field of experimental tuberculosis, Arima et al.[2] stated that sensitization of rabbits by killed tubercle bacilli was suppressed by 6-mercaptopurine. In contrast to the above papers reporting effectiveness of purine analogues, Tsukamura et al.[3] reported that adenine itself is able to modify infection of mice with tubercle bacilli. It was thus desirable to obtain further informations on biological effects of nucleic acid precursors. The purpose of the present paper is to report on the effect of orotic acid and 4-amino-5-imidazolecarboxamide orotate on infection of mice with tubercle bacilli.

Methods

CF_1 strain and dd-N strain of mice weighing 22 to 24g were used. Challenge was done by intravenous injection of *M. tuberculosis* $H_{37}Rv$ or *M. bovis* Ravenel, which were subcultured in Dubos TB broth (without tween) at 37°C for 7 days. Amount of challenge was 1mg moist weight of the $H_{37}Rv$ strain or 0.2mg moist weight of the Ravenel strain. For the purpose of immunity production, *M. bovis* BCG, subcultured on Ogawa egg medium for 3 weeks, was inoculated subcutaneously to mouse in a dose of 0.1mg moist weight.

Grade of infection was observed taking the number of viable challenge organisms recovered in the lungs and in the spleen of a mouse as indexes. To count the viable numbers, three mice were killed from each group, and the lungs and the spleens were measured of their weights. The organs of three mice were combined into one, respectively, and homogenized with a motor homogenizer adding 5 volumes of distilled water. The homogenate was then added with 5 volumes of 2% NaOH and again homogenized. The homogenate was diluted to give a series of 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions. Two-one hundredths m*l* samples of each dilution were inoculated with a spiral loop onto Ogawa egg medium and incubated at 37°C. After four weeks of incubation, the number of colonies was counted, and the number of viable challenge organisms recovered in a whole organ (lungs or spleen) was calculated.

Orotic acid was administered as orotic acid-dimethylamide (Orotonsan-S ; Ono Pharmaceutical Co.), which was supplied as a 20mg/m*l* solution. 4-Amino-5-imidazolecarboxamide orotate

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(Aicamin ; Fujisawa Pharmaceutical Co.) was supplied as a crystal form. This was dissolved in a 0.067 M phosphate buffer (pH 7.1) at a concentration of 10 mg/ml. Both agents were administered to mice by sabcutaneous injection.

Results

1. *Effect of Orotic acid-dimethylamide on experimental infection of mice with virulent tubercle bacilli*

The results are shown in Tables 1 and 2. Administration of orotic acid in daily doses of 0.1 mg to 2 mg for 6 or 8 weeks seemed to lower the infection with tubercle bacilli so far as viewed from bacteriological viewpoints. The best effect was obtained by daily administration of 2 mg (Table 1). Administration of daily doses 4 mg and 10 mg caused initial deterioration but followed later improvement (Table 1).

2. *Effect of 4-amino-imidazolecarboxamide orotate on experimental infection of mice with virulent tubercle bacilli*

The results are shown in Table 3. Administration of Aicamin in a daily dose of 0.2 mg or 0.5 mg retarded the time of occurrence of maximum increase of challenge organisms (Table 3).

3. *Effect of 4-amino-5-imidazolecarboxamide orotate on immunity production in mice by BCG vaccination*

After vaccination with BCG, the mice were divided into three groups. The first received injection of saline, the second subcutaneous injection of 0.2 mg of Aicamin, and the third that of 0.5 mg of Aicamin. The administration was made daily for 4 weeks. After 4 weeks, challenge was done by intravenous injection of *M. tuberculosis* $H_{37}Rv$. Two weeks and four weeks after challenge, the number of viable challenge organisms recovered in the lungs and the spleen were counted to observe the effect of vaccination. Post-vaccination administration of Aicamin in a daily dose of 0.2 mg per mouse seemed to give some good effect on production of immunity, whereas such administration in a dose of 0.5 mg per mouse seemed to counteract the immunity formation (Table 4).

Since orotic acid and Aicamin have practically no inhibitory effect on multiplication of tubercle bacilli, it is probable that the effects of these agents observed are due to modification of host tissues. By administration of the agents, the host tissues may become a state unfavorable for multiplication of tubercle bacilli. In case of the BCG vaccination also, modification of host tissues may concern with modified immunity formation.

Conclusion

Administration of orotic acid-dimethylamide could modify infection of mice with tubercle bacilli. Daily administration of this agent in doses 0.1 mg to 2 mg per mouse suppressed multiplication of tubercle bacilli in mouse organs (lungs and spleen). Administration of 4-amino-5-imidazolecarboxamide orotate also modified infection of mice with tubercle bacilli and immunity formation by BCG vaccination.

緒　言

Schwartz et al.[1] が 6-mercaptopurine による抗体産生の抑制を報告して以来，purine 類似物質による免疫抑制効果に関する報告が数多く発表された。結核に関する分野では，Arima et al.[2] が結核菌死菌によるウサギの感作が 6-mercaptopurine によつて抑制されることを報告している。上記の purine 誘導体の免疫抑制効果の報告に対して，Tsukamura et al.[3] は purine の一つ，adenine 自体にマウスの結核菌感染を修飾する効果を認

Table 1. Effect of Orotic Acid-dimethylamide (Orotonsan-S®) on Infection of Mice‡ with Tubercle Bacilli

Treatment after challenge	Organ	Viable counts per organ		
		Time after challenge		
		2 weeks	4 weeks	6 weeks
Control (not treated)	Lungs	358,000	1,568,600	358,050
	Spleen	445,450	379,250	73,012
Orotic acid 2 mg daily	Lungs	217	95,600	64,960
	Spleen	24,080	94,080	42,025
Orotic acid 4 mg daily	Lungs	2,962,500	3,417	580
	Spleen	7,273,750	47,190	15,010
Orotic acid 10 mg daily	Lungs	978,600	46	104,030
	Spleen	3,481,000	6,245	57,812

The viable challenge numbers are a mean of three mice.
Each mouse was challenged with 1 mg. moist weight (1.4×10^6 viable organisms) of *M. tuberculosis* $H_{37}Rv$. The mice challenged were divided into four groups and received the following post-challenge treatment until the end of the 6th week: (1) control received injection of saline; (2) administered daily with 2 mg of orotic acid; (3) administered daily with 4 mg of orotic acid; (4) administered daily with of 10 mg of orotic acid.
‡ CF_1 strain.

Table 2. Effect of Orotic Acid-dimethylamide (Orotonsan-S®) on Infection of Mice‡ with Tubercle Bacilli

Treatment after challenge	Organ	Viable counts per organ*		
		Time after challenge		
		4 weeks	6 weeks	8 weeks
Control	Lungs	141,705	7,350,000	16,512,000
	Spleen	11,685	71,957	56,240
Orotic acid 0.1 mg daily	Lungs	7,344	4,833,405	68,340
	Spleen	50,662	57,855	2,292
Orotic acid 0.5 mg daily	Lungs	89,460	441,000	3,177
	Spleen	5,767	15,375	1,116

* Mean of three mice.
Each mouse was challenged with 0.2 mg moist weight (2.0×10^6 viable organisms) of *M. bovis* Ravenel. After challenge, the mice were divided into three groups and received the following post-challenge treatment until the end of the 8th week: (1) control received injection of saline; (2) administered daily with 0.1 mg of orotic acid; (3) administered daily with 0.5 mg of orotic acid. Administration of orotic acid was made subcutaneously.
‡ CF_1 strain.

めた。このような核酸前駆物質の生物学的効果を更に研究するために，その後 orotic acid および 4-amino-5-imidazolecarboxamide orotate のマウス結核感染実験に対する効果を観察したので本報に報告する。

方　法

マウスは体重 22 ないし 24g の CF_1 系または dd-N 系マウスを用いた（雌雄混合）。

感染実験（攻撃実験）は *M. tuberculosis* $H_{37}Rv$ 株または *M. bovis* Ravenel 株の静注によつた。$H_{37}Rv$ 株および Ravenel 株ともに Dubos TB broth (Tween なし) 5 ml に 37°C 7 日間培養したものを比濁により，5 mg/ml または 1 mg/ml（いずれも湿菌量）に調製し，その 0.2 ml を尾静脈に注射した。したがつてマウス 1 匹への接種量は，$H_{37}Rv$ 株 1 mg，Ravenel 株 0.2 mg であつた。

マウスの免疫実験には *M. bovis* BCG 株湿菌量 0.1 mg 皮下接種を用いた。BCG は 1% 小川培地に 37°C 3 週培養したものを，ガラス玉コルベンで均一化し，生食水に 1 mg/ml の割合に浮遊させ，その 0.1 ml を皮下注射した。BCG 接種後，マウスを対照群と処置群に分け，4 週後に人型結核菌 $H_{37}Rv$ 株で攻撃して，その後は感染実験と同じに取扱つた。

感染後の経過の観察は，肺および脾の生菌数を数え，

Table 3. Effect of 4-amino-5-imidazolecarboxamide Orotate (Aicamin®) on Infection of Mice# with Tubercle Bacilli

Treatment after challenge	Organ	Viable counts per organ*					
		Time after challenge					
		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Control	Lungs	<31	235	1,545	848,700	40,970	<84
	Spleen	452	380	3,705	429,425	33,840	113
Aicamin 0.2mg	Lungs	<64	148	46	45,980	182,245	45,540
	Spleen	260	2,822	228	16,060	18,720	1,054
Aicamin 0.5mg	Lungs	246	1,011	1,820	2,544	1,131,500	12,584
	Spleen	242	1,886	3,260	19,380	71,910	2,369

* Mean of three mice.

Each mouse was challenged with 1mg moist weight (7.9×10^6 viable organisms) of *M. tuberculosis* $H_{37}Rv$. After challenge, the mice were divided into three groups and received the following post-challenge treatment: (1) control received injection of saline; (2) subcutaneously administered daily with 0.2mg Aicamin®; (3) subcutaneously administered daily with 0.5mg Aicamin®. The treatment was begun on the day of challenge and continued until the end of the 8th week.

dd-N strain.

Table 4. Effect of 4-amino-5-imidazolecarboxamide Orotate (Aicamin®) on Immunity Formation in Mice#

Treatment after BCG vaccination	Organ	Viable counts per organ*	
		Time after challenge	
		2 weeks	4 weeks
Control	Lungs	10,465	956
	Spleen	63,825	244
Aicamin 0.2mg	Lungs	66	2,160
	Spleen	1,609	965
Aicamin 0.5mg	Lungs	75,600	54,495
	Spleen	97,200	835,000

dd-N strain of mice.

* Mean of three mice.

Immunity formation against challenge with tubercle bacilli was observed taking the number of viable challenge organisms recovered in the lungs and the spleen as an index.

Mice (dd-N strain, 22 to 24g) were vaccinated subcutaneously with 0.1mg moist weight (1.8×10^5 viable organisms) of the BCG strain of *M. bovis*. The mice were then divided into three groups and received the following post-vaccination treatment for four weeks: (1) control received injection of saline every day; (2) the second received subcutaneous injection of Aicamin® solution, 0.2mg daily; (3) the third received subcutaneous injection of Aicamin® solution, 0.5mg daily. Four weeks after vaccination, the mice were challenged with intravenous injection of *M. tuberculosis* $H_{37}Rv$ (1.6×10^7 viable organisms). Two weeks and four weeks after challenge, the mice were sacrificed and the number of viable challenge organisms in the lungs and in the spleen were counted.

マウス1匹当りの肺または脾に含まれる攻撃菌の生菌数によつて感染の程度を表わした。生菌数の算定には，各群からマウス3匹をとつて殺し，肺および脾をとつておのおの秤量した後，3匹分の臓器を合して，これに5倍量の蒸留水を加えて motor homogenizer で均一化し，これに5倍量（臓器の5倍量）の 2% NaOH を加えて，臓器原液とした。この原液（結局，臓器にその10倍量の 1% NaOH を加えたことになる）を蒸留水で，10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} に希釈し，各希釈液（5種）から 0.02ml を 1% 小川培地に渦巻白金耳で接種した。1% 小川培地は各希釈液当り2本を用いた。接種した小川培地は 37℃ 4週培養した後，集落数を数え，集落数，希釈度および臓器重量から，マウス1匹の肺または脾当りの生菌数を算出した。

Orotic acid（以下 OA と略す）は，orotic acid-dimethylamide (Orotonsan-S®, 小野薬品) を用い，20 mg/ml の注射薬として調製された品を用いた。4-amino-5-imidazolecarboxamide orotate（以下 AICA と略す）は Aicamin® (藤沢薬品) を用いた。提供された純末を，0.067M 燐酸緩衝液 (pH 7.1) に 10mg/ml の割合に溶解して用いた。OA も AICA もともに皮下注射で投与した。

結　果

1. マウスの結核菌感染に対する orotic acid-dimethylamide (Orotonsan-S®) の効果

CF_1 系マウスを人型結核菌 $H_{37}Rv$ または牛型結核菌 Ravenel で攻撃した後，直ちに OA の注射を開始し，6週または8週続けた。結核菌感染に対する OA 投与の効果は，表1および表2に示すごとくで，OA の投与はマウスの結核感染に対して好影響をもたらすごとく思えた。特に 0.1mg ないし 2mg 毎日投与で好結果が得られた。1日量 4mg および 10mg の毎日投与では，感染初期にかえつて悪影響があつたが（肺および脾の生菌数が対照より多い），後には好転した。

2. マウスの結核菌感染に対する 4-amino-5-imidazolecarboxamide orotate (Aicamin®) の効果

dd-N 系マウスを人型結核菌 $H_{37}Rv$ で攻撃した後，マウスに AICA 0.2mg または 0.5mg を毎日注射し，対照と比較した。その結果は表3に示すごとくで，AICA 投与によつて生菌数が最大に達する peak の遅れがみられた。AICA の投与も結核感染を修飾するごとく思われた。

3. *M. bovis* BCG によるマウス免疫に対する 4-amino-5-imidazolecarboxamide orotate (Aicamin®) の効果

マウス (dd-N 系) に BCG 0.1mg の皮下接種を行なつた後に，マウスを3群に分けて次のごとく処置した。（1）第1群は対照で生食水 0.2m*l* を毎日注射，（2）第2群には AICA 0.2mg を毎日皮下注射，（3）第3群には AICA 0.5mg を毎日皮下注射。上下の処置を4週間続けた後に，マウスに人型結核菌 $H_{37}Rv$ 株を静注して攻撃し，その2週および4週後に肺および脾の生菌数を数えた。結果は表4のごとくである。

BCG 接種後，攻撃までの期間，すなわち BCG によつて免疫が形成される時期における AICA の投与が免疫形成にいかなる影響を示すかをみたわけであるが，表4に示すように，AICA 0.2mg の投与はたいした影響がないか，または若干好影響があるかに思われ，AICA 0.5mg の投与はむしろ悪影響があるかにみえた。

以上の結核菌感染および BCG 接種後の免疫形成に及ぼす OA および AICA の作用機序としては直接的な抗菌作用は考えにくい。おそらく OA または AICA が生体組織の状態を修飾して結核菌の発育に不都合な状態を作り出すものと考えてよかろう。

以上の観察はマウスの実験結核に関するものであり，観察期間も十分とはいえないから，これから人間の場合や他の動物の場合を軽々に類推するわけにはゆかない。しかし OA や AICA のごとき核酸前駆物質の投与が，マウスの結核菌感染を修飾しうることは注目すべきことと思われる。

結　論

Orotic acid-dimethylamide (Orotonsan-S®) の注射はマウスの結核菌感染を修飾するごとく思われた。1日量 0.1mg ないし 2mg の毎日注射は，肺および脾の攻撃菌生菌数を指標としてみる限り，感染経過に好影響を及ぼすごとく思われた。

4-amino-5-imidazolecarboxamide orotate (Aicamin®) の注射もマウスの結核感染に影響を及ぼす。1日量 0.2mg または 0.5mg の注射で，肺および脾の攻撃菌生菌数 peak の時期が遅延するごとく思われた。また BCG 接種による免疫形成にも影響がみられた。

上述の Orotic acid-dimethylamide および 4-amino-5-imidazolecarboxamide orotate による感染の修飾は，宿主組織に対する修飾効果に基づくものと想像される。

文　献

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