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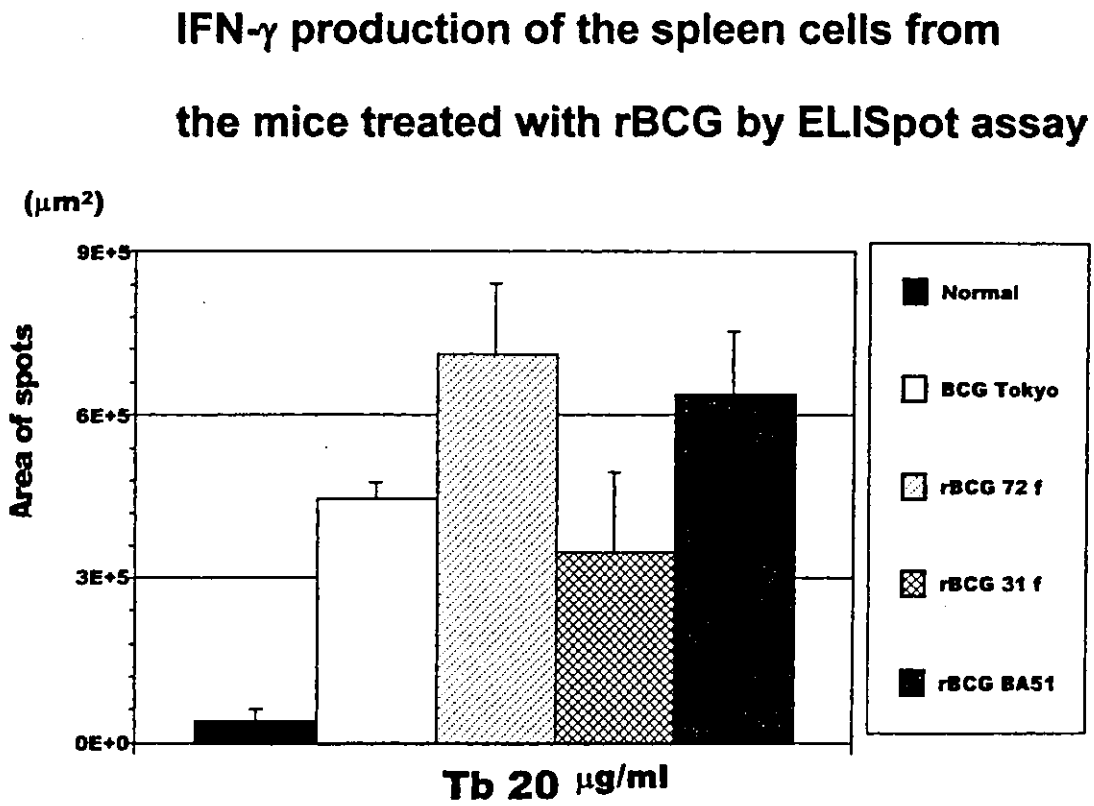
(Fig 1)



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▷薬剤耐性菌感染症の現状と検査室の対応(1)◁

結核菌感染症

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シンポジウム 1

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Present Trends of Drug-resistant Tuberculosis and How to Manage It by Mycobacterial Laboratories

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Global, domestic, and local trends of drug-resistant tuberculosis and how to manage increase in the resistance by mycobacterial laboratories were discussed based on literatures and our own data. At first, how to make drug-resistant tuberculosis was explained. Genetic drug-resistant bacteria were emerged spontaneously by mutation of the genome and were selected by inadequate treatment (mono-therapy or functional mono-therapy): acquired drug resistance (single, and then multi-drug resistance). In a mean time, some people were infected with the drug-resistant bacteria from the beginning and a part of them developed active disease: primary drug resistance (single or multi-drug resistance). Estonia, Latvia, Iran, and some part of Russia, China, and India were reported to be the most endemic region of drug resistant and multi-drug resistant tuberculosis in the world. The rate of primary resistance in Japan was as high as the median of the world, but the rate of acquired resistance was almost twice of the median. Since delays in reporting of the drug resistance from the laboratory seemed one of reasons for the inadequate treatment, drug susceptibility testing should be more rapid than usual, by using liquid media such as BACTEC MGIT 960 ™ system, or gene analysis.

[Rinsho Byori 50 : 847～852, 2002]

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【Key Words】 *M. tuberculosis*（結核菌），drug resistance（薬剤耐性），drug susceptibility test（薬剤感受性試験）

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戦前まで死に至る病として世界的に恐れられていた結核は，戦後ストレプトマイシン(SM)を嚆矢とする抗結核薬剤の相次ぐ開発と社会・経済環境の改善により，先進国で死亡率・罹患率の急速な低下を認めた。そのため結核は早晩撲滅されるとの楽観論が1980年代には大勢を占めるまでになった。しかし発展途上国での急速な都市化の進行と先進国でのHIV感染症の蔓延が，先に述べた楽観論に基づく結核対策の手抜きと相まって，世界的な結核の再興を引き起こす結果となった。特にHIV感染者における多剤耐性結核の合併は短期間に高い死亡率を示す事で1990年代初頭のマスコミを大いににぎわした事は記憶に新しい。その後WHOを中心とする調査により結核菌の薬剤耐性がHIV感染の有無によらず世界的に進行している事が指摘されている[1)2)]。結核が克服された大きな要因が有効な化学療法レジーメンの開発であった事に異論はなく，薬剤耐性結核の出現と蔓延は今後の結核対策に大きな影を投げかけている。本稿では，結核菌薬剤耐性のメカニズム，世界と日本の現状，薬剤耐性結核の克服における検査室の対応につき，文献と当院での経験に基づき概説した。

I. 結核菌薬剤耐性のメカニズム

結核菌の薬剤耐性はゲノムDNAの突然変異により生じる。幸いこの突然変異は薬剤毎に独立しているため，1回の変異で複数薬剤の耐性が同時に生じる事はない。**Table 1**に主要抗結核薬剤の耐性遺伝子と同遺伝子の変異で説明可能な薬剤耐性の率を呈示した[3)]。リファンピシン(RFP)とピラジナマイド(PZA)以外の薬剤には複数の耐性遺伝子が存在しているため，薬剤耐性を遺伝子工学的に検出する方法の開発と一般的な普及を困難にしている。薬剤耐性遺伝子が発見される以前より，薬剤毎の耐性菌の出現頻度は実験データからつぎの様に推測されている：RFP $1/10^8$，イソニアジド(INH) $1/10^6$，SM $1/10^7$，エタンブトール(EB) $1/10^5$，PZA $1/10^6$。一般的に耐性菌は主要な遺伝子に突然変異を持っているため，感受性菌と比べると患者体内での生存には不利である。従ってたまたま生じた耐性菌が感受性菌を押し退けて多数を占める事は未治療例では考えられない。しかしある薬剤で治療中にその薬剤の耐性菌が生じた場合，感受性菌のほとんどが殺菌されてしまうため，耐性菌のみが増殖し早晩大多数を占める事になる。一方従来からの研究でヒト肺結核病巣中の菌量の概略は，空洞中に 10^8～10^9，結節中に 10^5～10^6 と判明している[3)]。従って空洞を持つ症例の場合，最初から100～1000のINH耐性菌が存在している計算となり，INHのみを投与した場合1ヶ月をまたずにINH耐性結核となる危険性が高い事が容易に推測される。この際INHとRFPが同時に投与されておれば，両薬剤に耐性の結核菌がたまたま生じる確率は 10^{14} 分の1と計算されるので，耐性菌が選択的に増殖する可能性は患者100万人に1人程度と臨床的には無視できる数字となる。結核治療の原則が多剤併用療法である一つの目的は，このような耐性菌の誘導を防止する事にある。結核菌に感染しているが発病していないヒトの一部にINHの単独投与による化学予防が行われている。現在までの膨大な臨床データから，予防投与後の発病者にINH耐性菌の出現はほとんどない事が判明している。これは感染・未発病者体内の結核菌量が100万より

Table 1 Frequency of mutations in clinical isolates of *M. tuberculosis*

Drug	Resistant Gene	Function of the Gene	Altered Sequence
INH	*katG*	catalase-peroxidase	50%
	inhA	enoyl-acyl reductase	20%
	ahpC	alkyl hydroxyperoxidase	15%
RFP	*rpoB*	RNA polymerase β subunit	95%<
SM	*rpsL*	ribosomal protein S12	50%
	rrs	16S ribosomal RNA	10%
EB	*embB*	arabinosyl transferase	47～65%
PZA	*pncA*	pyrazinamidase	72～97%

INH : Isoniazid, RFP : Rifampicin, SM : Streptomycin, EB : Ethambutol, PZA : Pyrazinamide

大幅に少ないため，INH耐性菌の突然変異による出現がほとんどないためと考えられている。さてINHの単独投与により生じたINH耐性結核例に，次いでRFPを加えた場合，同様の機序でさらにRFP耐性も獲得し，多剤耐性結核となる事は容易に理解される。ちなみにWHOはINHとRFP両剤耐性以上の結核菌を多剤耐性結核菌と定義している。本邦の臨床で時々見られるのは，初回INH・SM耐性例にINH・RFP・SMの標準療法を行い，感受性の確認を怠りまたは無視して同じ治療を漫然と続けたために2〜3ヶ月ほどでRFPにも耐性を獲得するケースである[4]。主治医としては多剤併用療法をしているつもりであり，その上初期2〜3ヶ月間は症状，画像，検査所見の改善傾向が認められるために，よもや多剤耐性結核を作っているとは思わないのである。薬剤感受性結果の判明が遅い事もこのような治療のミスを生む要因となっている事は明らかであり，液体培地等を用いた感受性検査の迅速化をさらに推進する必要がある。

II. 初回薬剤耐性と獲得薬剤耐性

ある地域で各種薬剤を用いた結核の治療が普及していくと，単剤投与による耐性菌の出現が早晩認められる。さらに有効薬を一つずつ加えるミスを重ねる事で耐性薬剤数はどんどん増加していく結果となる(**Fig. 1**)。このような単剤投与により，最初は全剤感受性であった菌が耐性化した場合を獲得薬剤耐性と呼んでいる。多剤耐性菌の治療は基本的に難しく長期を要し，患者が排菌している期間も長い。従ってある地域に獲得薬剤耐性菌による結核患者が増加してくると，最初から耐性菌に感染し発病する患者が出現するようになる(初回薬剤耐性)[5]。実際は，初めて化学療法を受ける患者(初回治療例)が耐性の場合初回耐性と，一方化学療法を既に受けている患者が再発し(再治療例)その際耐性であった場合を獲得耐性と判断している。一般的に初回薬剤耐性率はその地域の公衆衛生のレベルを，一方獲得薬剤耐性率は結核の医療レベルを示していると言われている。

III. 世界の結核菌薬剤耐性の現状

1996年から1999年まで，世界58地域での結核菌薬剤耐性を調査したWHOとInternational Union Against Tuberculosis and Lung Diseaseの共同調査のデータの概要を以下に述べる[2]。初回治療例では，INH・RFP・SM・EBの主要4薬剤のどれかに対する耐性率の中間値は10.7%(1.7〜36.9)，INH・RFP両剤耐性以上の多剤耐性菌の割合の中間値は1.0%(0〜14.1)，INH耐性率の中間値は6.2%，RFPは同1.2%，SMは同5.2%，EBは同0.6%と報告されている。一方再治療例では，4薬剤どれかに対する耐性率の中間値は23.3%(0〜93.8)，多剤耐性菌の割合の中間値は9.3%(0-48.2)，INH耐性率の中間値19.6%，RFPは同12.0%，SMは同12.4%，EBは同5.9%となっている。再治療例で耐性率，特に多剤耐性率が格段に上昇することが分かる。エストニア，ラトビア，イランの他，ロシア，中国，インド，アフリカ諸国の一部地域で特に耐性菌の割合が高い。初回多剤耐性率ではエストニアの14.1%，中国河南省の10.8%，ロシアIvanova Oblastの9.0%，イランの8.1%が特に高く，一方再治療例での多剤耐性率ではイランの48.2%，エストニアの37.8%，中国浙江省の35.0%，同河南省の34.4%が特に目立った数字である。さらにこれらの地域での耐性率は調査した範囲では年々上昇傾向を示しているという。一方欧米先進国の耐性率は概して低くかつ一般的に低下傾向を示している。世界の人口の多くを占める中国やインドで結核菌の薬剤耐性が進行していることは，今後世界の結核対策に大きな悪影響を与える事は間違いない。

IV. 日本における結核菌薬剤耐性の現状

日本の主要な結核治療施設入院例薬剤耐性率を5年毎に調査している結核療法研究協議会の1997年の調査結果が昨年発表された[6]。以下その概要を述べる。初回治療例の主要4薬剤どれかに対する耐性率は10.3%，多剤耐性率は0.8%，INH耐性率4.4%，RFP 1.4%，SM 7.5%，EB 0.4%であった。これらの数字は先に述べた世界の中間値に近い値である。一方再治療例では，4薬剤どれかの耐性率が42.4%，多剤耐性率が19.7%，INH耐性率33.0%，RFP 21.6%，SM 24.2%，EB 15.2%と格段に上昇し，世界の中間値の2倍に近い数字となっている。日本で特に再治療例での耐性率が高い正確な要因はいまだ明らかにはされていないが，先に述べた一般的な見解から考えると，本邦の結核に対する医療レベルが世界の平均と比べて低いと結論せざるを得ない。

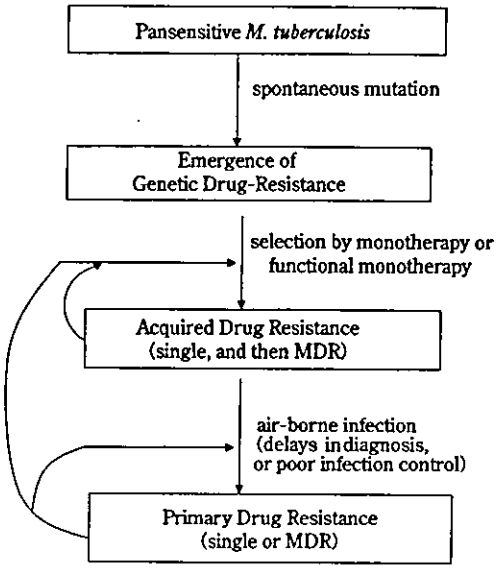

Figure 1 Acquired drug resistance and primary drug resistance
MDR : multi-drug resistant

V. 薬剤感受性検査の新しい方法

現在世界で一般に用いられている薬剤感受性検査の方法を**Table 2**に掲示した。全世界的には卵培地を用いる比率法が最も一般的である。また米国では従来寒天培地を用いる比率法が主流であったが，近年液体培地を用いる方法に転換されつつある。我が国では長く小川培地を用いる絶対濃度法が用いられてきた。しかしこの方法には以下に示すような多くの欠点が指摘されている：1)1薬剤に複数の濃度が設定されている，2)完全耐性と不完全耐性の二つの判断基準がある，3)薬剤濃度が世界の標準と異なっている，4)結果が判明するのに培養が陽性になってから最低でも3週間，場合によっては5週間もかかる。1)，2)のため結果の解釈が複雑で，実際患者に投与するべきかどうかの判断が一般臨床医には難しくなっている。また3)のため，我が国のデータが国際的には通用しない事態となってしまった[7]。そのため1997年日本結核病学会の抗酸菌検査法検討委員会は小川培地を用いる比率法の採用と薬剤濃度の国際標準化を柱とする新しい薬剤感受性検査の提言を行った[8]。また2000年に発行された新結核菌検査指針でも新しい方法を標準法として推奨している。最近になりようやく新しい方法が一般臨床に普及しつつあるが，旧来の絶対濃度法を用いている施設も未だに多い。**Table 3**と**Table 4**に新しい方法と旧来の方法の実例とその臨床的な解釈を例示しておいたので参考にされたい。さて新しい薬剤感受性検査法の導入は1)2)3)の問題点を解消したが，結果の判明が遅い点は解消されていない。迅速性を求めて，液体培地を用いた薬剤感受性検査[9]や薬剤耐性遺伝子の変異を検出する方法が各種開発されている。当院では2001年2月より比率法を導入し，さらに本年8月より液体培地による感受性検査(BACTEC MGIT 960)も併用している。新規に結核菌と同定された株はまずMGITによる主要4剤とPZAの感受性検査を行い，4薬剤のどれかに耐性があった場合は自動的に比率法を実施し，二次薬とLVFXの感受性を検

Table 2 Methods of drug susceptibility testing of *M. tuberculosis*

1. Absolute concentration method using Ogawa-egg media
2. Proportion method using egg media
3. Proportion method using agar-based media
4. Using liquid media (BACTEC MGIT 960 , Broth MIC etc.)
5. Gene analysis (PCR-direct sequence, line probe assay, hetero-duplex, DNA chip etc.)

Table 3 Example of drug susceptibility testing by absolute concentration method

	INH			RFP		SM		EB		KM		C
mg/ml	0.1	*1*	10	10	*50*	*20*	200	2.5	*5*	*25*	100	
CFU	3+	3+	3+	45	0	100	0	3+	0	4	0	3+

C : control, 3+ = 500～2000 CFU, 2+ = 200～500 CFU
Italic figures indicate drug concentration for clinical break-point
The result indicates that RFP and KM are expected to be effective clinically, but neither INH, SM nor EB is expected to be effective against the bacteria.

Table 4 Example of drug susceptibility testing by proportion method

	INH0.2*	INH1.0	RFP40	EB2.5	SM10	KM20	LVFX1.0	C	C/100†
CFU	65	0	2+	115	90	50	0	4+	100
Result	S	S	R	R	S	S	S		

*Drug concentration (mg/ml), †C=control, C/100=control CFU/100
LVFX=Levofloxacin, S=susceptible, R=resistant
Drug with CFU<CFU of C/100 is judged to be effective clinically against the bacteria.
Result of INH 0.2 is used unless multi-drug resistant (MDR) cases.
In MDR case, INH can be used clinically if the result of INH1.0 is susceptible.

討するプロトコールである。培養と薬剤感受性検査ともにMGITを用いる事で喀痰提出から2〜3週間で薬剤感受性の結果まで判明する時代が到来している。

VI. 新しい抗酸菌検査と精度管理

1990年代になり核酸の相同性を用いる同定キットを皮切りに，核酸増幅法を用いる迅速検出キット，液体培地による培養システム，新しい薬剤感受性試験，抗抗酸菌抗体の検出キット等，新しい抗酸菌検査が次々と出現したため検査室の仕事量はうなぎ登りとなり，また多くの臨床医は新しい検査の臨床的意義に関して少なからぬ混乱を経験した。確かに新しい検査手技は抗酸菌検査に革命的なインパクトを与えたが，現在までのところ結核医療の効率化に目に見えた貢献はしていない。液体培地の導入は菌検出を迅速化することで結核患者の入院期間を短縮するはずであったが，培養陰性を退院の条件にしている本邦の多くの結核病院では，その感度の高さから却って入院期間が延びるという皮肉な現象も出現している。一方新しい検査法の感度の高さと迅速性は検査室での精度管理の重要性を増加させている。集菌蛍光塗抹法，核酸増幅法，薬剤感受性試験は擬陽性や過大評価が生じやすく，かつその結果が患者マネージメントに大きな影響を与えるため，精度管理が特に重要である。精度管理の方法として標準的な検体をプールしておき検査の正確性を半年毎にチェックする等の方法が考えられるが，今後臨床検査医学会や結核病学会が主体となり標準的な方法を提言していく必要があろう。また最終的な検査の妥当性は臨床の経過との一致で検証されるのだが，結核の場合経過が緩慢でありかつ多剤併用療法が行われるため，この検証が行いにくいのも難点となっている。各種検査の結果を定期的にまとめて既存の報告と比較するとともに，臨床サイドと検査サイドの連絡を密にすることで異常なもしくは臨床経過とあわない検査結果の早期発見に努める必要がある。

VII. 終わりに

どんなに感度・特異度の高い検査でも，検査室で正しく実施されかつ臨床で正しく解釈されなければ宝の持ち腐れである。感染症，特に抗酸菌症は検査結果が直接に診断と治療に結びつくため，臨床医は検査法の概要と結果の解釈に精通していなければならないし，検査サイドも臨床の概要を知る必要がある。さらに検査室と臨床医のコミュニケーションを密にする事が，効率良く経済的な検査の実施のみならず，精度管理上も極めて重要な事を重ねて強調したい。そのため自施設で微生物検査が可能である事が，結核に限らず感染症専門施設としての必要条件であると考えている。

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Interferon Regulatory Factor 1 in Mycobacterial Infection

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***Abstract*: In order to understand the role of IRF-1 in the development of murine tuberculosis *in vivo*, IRF-1 knockout mice were infected with *Mycobacterium tuberculosis* by placing them in the exposure chamber of an airborne infection apparatus. These knockout mice developed multifocal necrotic lesions in the lung, liver and spleen tissues and died of disseminated tuberculosis within 43 days of infection. Compared with the levels in wild-type mice, the pulmonary inducible NO synthase (iNOS) mRNA expression level was significantly lower, but IL-18 and IL-6 mRNA levels were higher. There was no statistically significant difference in the expression of IFN-γ and TNF-α mRNA between the IRF-1 knockout and wild-type mice. IRF-1 is indirectly responsible for iNOS mRNA expression and plays an important role in the pathogenesis of murine tuberculosis.**

***Key words*: IRF-1, *Mycobacterium tuberculosis*, Aerosol infection, Knockout mouse**

Interferon regulatory factors (IRFs) are a family of 9 transcription factors possessing a novel helix-turn-helix DNA-binding motif (26). IRF-1 and IRF-2 mRNAs are both expressed in a variety of cell types. It has been reported that IRF-1 can activate IFN-α/β promoters (7, 8). IRF-1 functions as a transcriptional activator, while IRF-2 functions as a transcriptional repressor for the IFN-α/β genes (8, 10). IRF-1 induces the transcription of IFN-α/β genes via double-stranded RNA and induces antiviral activity by IFN-α/β and IFN-γ (15, 18). Several experiments using IRF-1 knockout (KO) mice have shown that the induction of inducible nitric oxide synthase (iNOS) gene expression disappears in macrophages stimulated by IFN-γ (IFN-γ-stimulated inducer of iNOS expression) (13). Incomplete differentiation of Th1 cells and the disappearance of NK cell activity have also been reported (19). Thus, it is suggested that IRF-1 is involved in regulation of the biodefense system.

On the other hand, Manca et al. suggested that IFN-α/β is involved in infection with a *Mycobacterium tuberculosis* clinical isolate (17); IFN-α mRNA levels were significantly higher in the lungs of the infected mice. Kamijo et al. reported that macrophages from IRF-1 KO mice produced little or no nitric oxide (NO) and synthesized barely detectable iNOS mRNA in response to infection with *Mycobacterium bovis* (13). Infection with *M. bovis* Bacille bilié de Calmette-Guérin (BCG) was more severe in IRF-1 KO mice than in wild-type (WT) mice. Although IFN-γ plays a pivotal role in defense against mycobacterial infection, IFN-α/β and IRF-1 activating IFN-α/β promoters also seem to be important in the defense against mycobacterial infection. These findings prompted us to explore the role of IRF-1 in mycobacterial infection *in vivo*. We report here that IRF-1 is very important for preventing the development of murine tuberculosis.

Materials and Methods

Animals. Six-week-old C57BL/6 wild-type mice were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan) and C57BL/6 IRF-1 KO mice (B6.129-*Irf1*^{tm1Mak}) were purchased from Jackson Laboratory (Bar Harbor, Me., U.S.A.) (18). These KO mice showed no developmental abnormalities. All mice were housed in a biosafety level 3 facility and fed mouse chow and water *ad libitum*.

Experimental infections. The experimental procedures were in accordance with the ARVO resolution on the use of animals in research. Permission (No. 20013) to experiment on animals was given by the Animal Experiment Committee in the Research Institute of Tuberculosis. A virulent Kurono strain of *Mycobac-*

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Abbreviations: IL, interleukin; IRF, interferon regulatory factor; KO, knockout; WT, wild-type.

terium tuberculosis (ATCC 358121) was grown in Middlebrook 7H9 broth for two weeks, and then filtered with a sterile acrodisc syringe filter (Pall Corp., Ann Arbor, Mich., U.S.A.) with a pore size of 5.0 μm. Aliquots of the bacterial filtrate were stored at −80 C. Mice were infected via the airborne route by placing them into the exposure chamber of a Glas-Col aerosol generator (Glas-Col, Inc., Terre Haute, Ind., U.S.A.). The nebulizer compartment was filled with 5 ml of a suspension containing 10^6 colony-forming units (CFUs) of Kurono tubercle bacilli so that about 1,000 bacteria could be deposited in the lung of each animal (27).

CFU assay. At 1, 3, 5 and 7 weeks after infection, the mice were anesthetized with pentobarbital sodium, the abdominal cavity was incised and exsanguination was achieved by splenectomy. Lungs, spleens and livers were excised and weighed. The left lobe of each lung and a section of spleen were weighed separately and used to evaluate *in vivo* growth of the mycobacteria. The lung and spleen tissues were homogenized with a mortar and pestle, and 1 ml of sterile saline was added. One-hundred microliters of the homogenate was picked up and plated in 10-fold serial dilutions on 1% (v/v) Ogawa's egg media. The colonies that grew on the media were counted after a 4-week incubation at 37 C.

RT-PCR. The right lobe of the lungs (three mice each) were used to a perform reverse-transcription-polymerase chain reaction (RT-PCR) analysis for mRNA expression of several cytokines and iNOS in these organs during TB infection. The tissue samples were snap-frozen in liquid nitrogen, and stored at −85 C. RNA extraction was performed as described previously (22, 23). Briefly, frozen tissues were homogenized with a microcentrifuge tube and a tip-closed 1-ml pipet in liquid nitrogen. The homogenates were treated with a total RNA isolation reagent, TRIzol™ Reagent (GIBCO BRL), according to the manufacturer's instructions. After the RNA was isolated, the total RNA concentration was measured, the RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase (GIBCO BRL), and agarose gel electrophoresis was performed.

A polymerase chain reaction was performed with gene-specific primer sets for β-actin, IFN-α, IFN-β, IFN-γ, TNF-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-18, TGF-β, and iNOS. The primer sequences and PCR product sizes were as follows: for β-actin, 5′-TGT GAT GGT GGG AAT GGG TCA G-3′ (sense) and 5′-TTT GAT GTC ACG CAC GAT TTC C-3′ (antisense), 514 bp; for IFN-α, 5′-ATGGCTAG-GCTCTGTGCTTTCCT-3′ (sense) and 5′-AGGGCTCT-CCAGAYTTCTGCTCTG-3′ (antisense), 524 bp; for IFN-β, 5′-CATCAACTATAAGCAGCTCCA-3′ (sense) and 5′-TTCAAGTGGAGAGCAGTTGAG-3′ (antisense), 354 bp; for IFN-γ, 5′-TAC TGC CAC GGC ACA GTC ATT GAA-3′ (sense) and 5′-GCA GCG ACT CCT TTT CCG CTT CCT-3′ (antisense), 405 bp; for TNF-α, 5′-ATG AGC ACA GAA AGC ATG ATC-3′ (sense) and 5′-TAC AGG CTT GTC ACT CGA ATT-3′, 276 bp; for IL-1β, 5′-CAG GAT GAG GAC ATG AGC ACC-3′ (sense) and 5′-CTC TGC AGA CTC AAA CTC CAC-3′ (antisense), 447 bp; for IL-2, 5′-CTT CAA GCT CCA CTT CAA GCT-3′ (sense) and 5′-CCA TCT CCT CAG AAA GTC CAC-3′ (antisense), 400 bp; for IL-4, 5′-ACGGA-GATGGATGTGCCAAACGTC-3′ (sense) and 5′-CGAGTAATCCATTTGCATGATGC-3′ (antisense), 279 bp; for IL-6, 5′-CAT CCA GTT GCC TTC TTG GGA-3′ (sense) and 3′-CAT TGG GAA ATT GGG GTA GGA AG-3′ (antisense), 463 bp; for IL-10, 5′-CCA GTT TTA CCT GGT AGA AGT GAT -3′ (sense) and 5′-TGT CTA GGT CCT GGA GTC CAG CAG-3′ (antisense), 324 bp; for IL-12p40, 5′-ATC TCC TGG TTT GCC ATC GTT TTG-3′ (sense) and 5′-TCC CTT TGG TCC AGT GTG ACC TTC-3′ (antisense), 527 bp; for IL-18, 5′-ACTGTACAACCGCAGTAATACGG-3′ (sense) and 5′-TCCATCTTGTTGTGTCCTGG-3′ (antisense), 319 bp; for TGF-β, 5′-CGG GGC GAC CTG GGC ACC ATC CAT GAC-3′ (sense) and 5′-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3′ (antisense), 371 bp; and for iNOS, 5′-TGG GAA TGG AGA CTG TCC CAG-3′ (sense) and 5′-GGG ATC TGA ATG TGA TGT TTG-3′ (antisense), 306 bp. Amplification was performed with a DNA thermal cycler 480 (Perkin-Elmer Cetus). Annealing temperature varied from 56 to 68 C depending on the primer sets used. The cycle number also varied from 23 to 40. Ten microliters of each PCR product was electrophoresed in a 4% (w/v) agarose and NuSieve GTG (1:3) gel and visualized using ethidium bromide staining. The β-actin RNA from the lung tissues was used as an internal control for size comparison in the RT-PCR analysis. Thereafter, densitometric analysis of the RT-PCR results was carried out using NIH image software.

Light and electron microscopic examination. For light microscopic examination, the right middle lobe of each lung was excised and fixed with a 20% (v/v) formalin-buffered methanol solution, Mildform 20NM (containing 8% (v/v) formaldehyde and 20% (v/v) methanol; Wako Pure Chemical Co., Osaka, Japan), dehydrated with a graded ethanol series, treated with xylene and embedded in paraffin. Five-micrometer-thick sections were cut from each paraffin block and stained using either the hematoxylin and eosin or Ziehl-Neelsen method.

For electron microscopy, the right lower lobe of each animal's lung was fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), at 4 C overnight,

washed three times with cold PB, post-fixed with 1% (w/v) osmium tetroxide in PB at 4 C for 1 hr, dehydrated with a graded ethanol series containing 10% (v/v) methanol and embedded in Spurr's low-viscosity resin. Ultrathin sections were cut using a Reichert Ultracut and stained with uranyl acetate and Sato's lead solution. The stained ultrathin sections were examined with a JEM-1230 electron microscope (JEOL, Tokyo) (27).

Statistical methods. The results were analyzed by Student's *t*-test for comparison between the two groups and by nonparametric equivalents of ANOVA (analysis of variance) for multiple comparison.

Results

Mycobacterial Burden in the Lung and Spleen of IRF-1 KO Mice

IRF-1 KO mice died of disseminated tuberculosis by

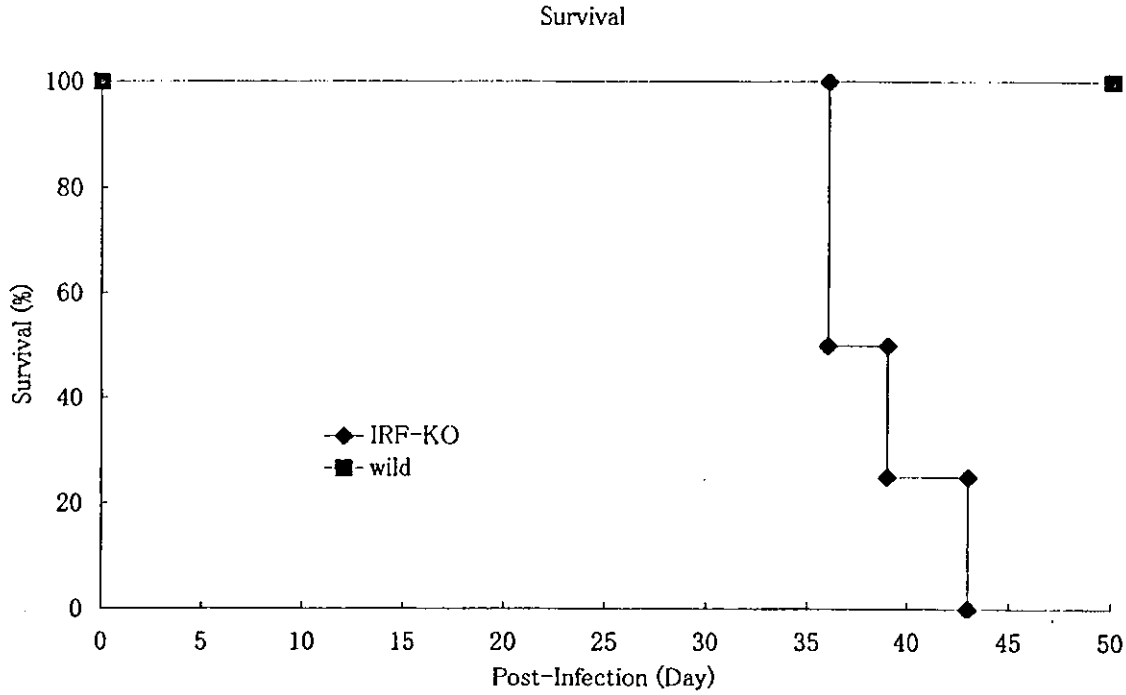

Fig. 1. Survival curves of mice infected with *M. tuberculosis* Kurono strain. The data presented are from two separate experiments with 10 mice in each group.

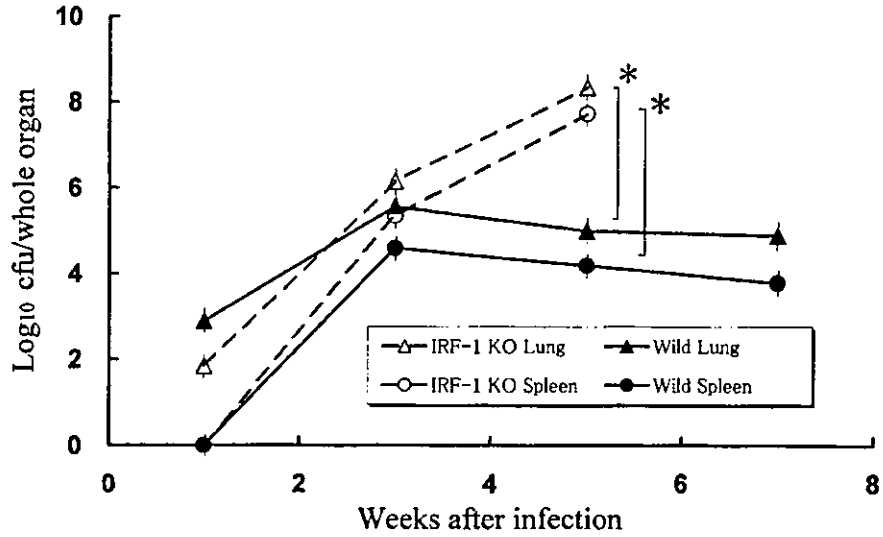

Fig. 2. CFUs in lung and spleen tissues of IRF-1 KO and WT mice (10 mice each) exposed to 10^6 CFUs of *M. tuberculosis* Kurono strain by the airborne route. * Indicates significant difference at $P<0.01$.

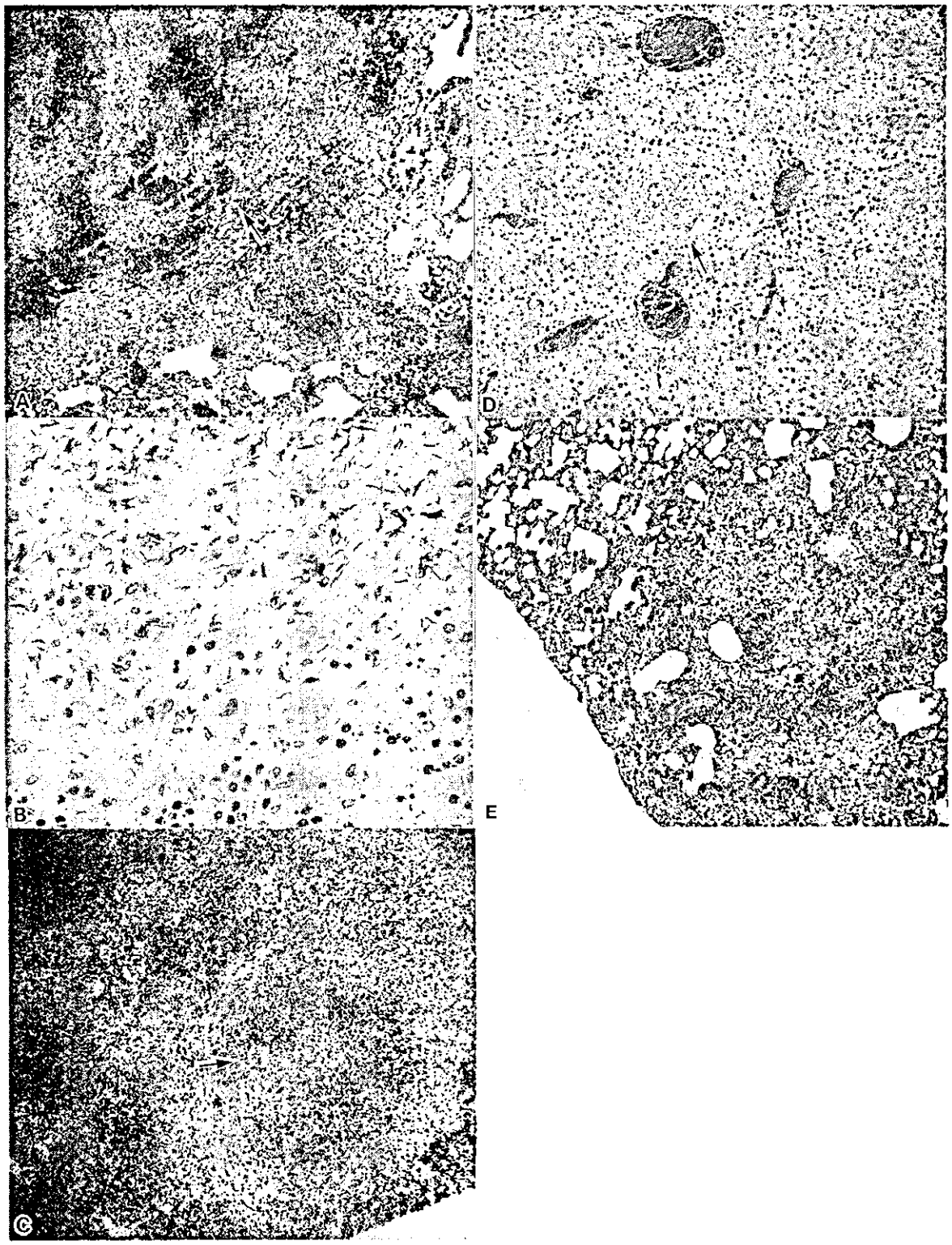

Fig. 3. Histopathology of the infected mice. Mice were sacrificed five weeks after infection with *M. tuberculosis* Kurono strain, and formalin-fixed sections were stained with hematoxylin and eosin (A, C, D and E) or Ziehl-Neelsen stain for acid-fast bacilli (B). (A) Pulmonary tissue from an IRF-1 KO mouse infected with Kurono strain. Magnification, ×135. (B) Pulmonary tissue from an IRF-1 KO mouse stained by the Ziehl-Neelsen method. Magnification, ×600. (C) Spleen tissue from an IRF-1 KO mouse. Magnification, ×135. (D) Liver tissue from an IRF-1 KO mouse. Magnification, ×135. (E) Pulmonary tissue from a WT mouse infected with Kurono strain. Magnification, ×135. Necrotic lesions are indicated by the arrow (→).

the 43rd day after infection, whereas WT mice survived until they were killed on day 43 (Fig. 1). Three weeks after infection, there was no significant difference in lung and spleen CFUs between IRF-1 KO mice and C57BL/6 WT mice; both groups had approximately 10^5 CFUs in the lungs. However, after five weeks postinfection, many IRF-1 KO mice succumbed to the mycobacterial infection. CFU counts of the lung and spleen tissues exceeded 10^7 CFUs. At this time point, there were statistically significant differences in both lung and spleen CFU counts between WT and IRF-1 KO mice ($P<0.01$) (Fig. 2).

Light and Electron Microscopic Observation of Infected Lungs

Consistent with the CFU changes, the histopatholog-

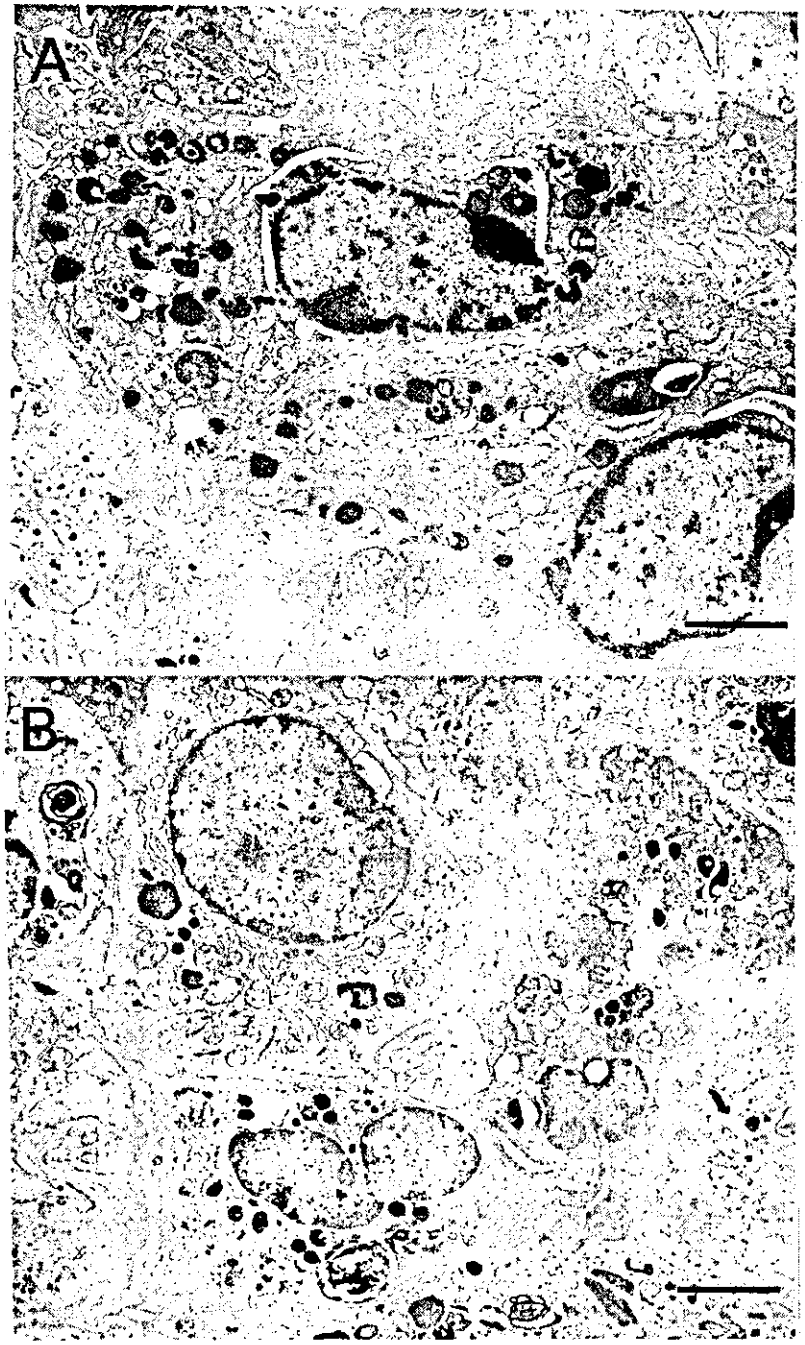

Fig. 4. Electron micrographs of lung tissue from WT (A) and IRF-1 KO (B) mice infected with Kurono strain, obtained five weeks after airborne infection. Many phagosomes ingest tubercle bacilli (B). Magnification, ×7,000. —= 2 μm.

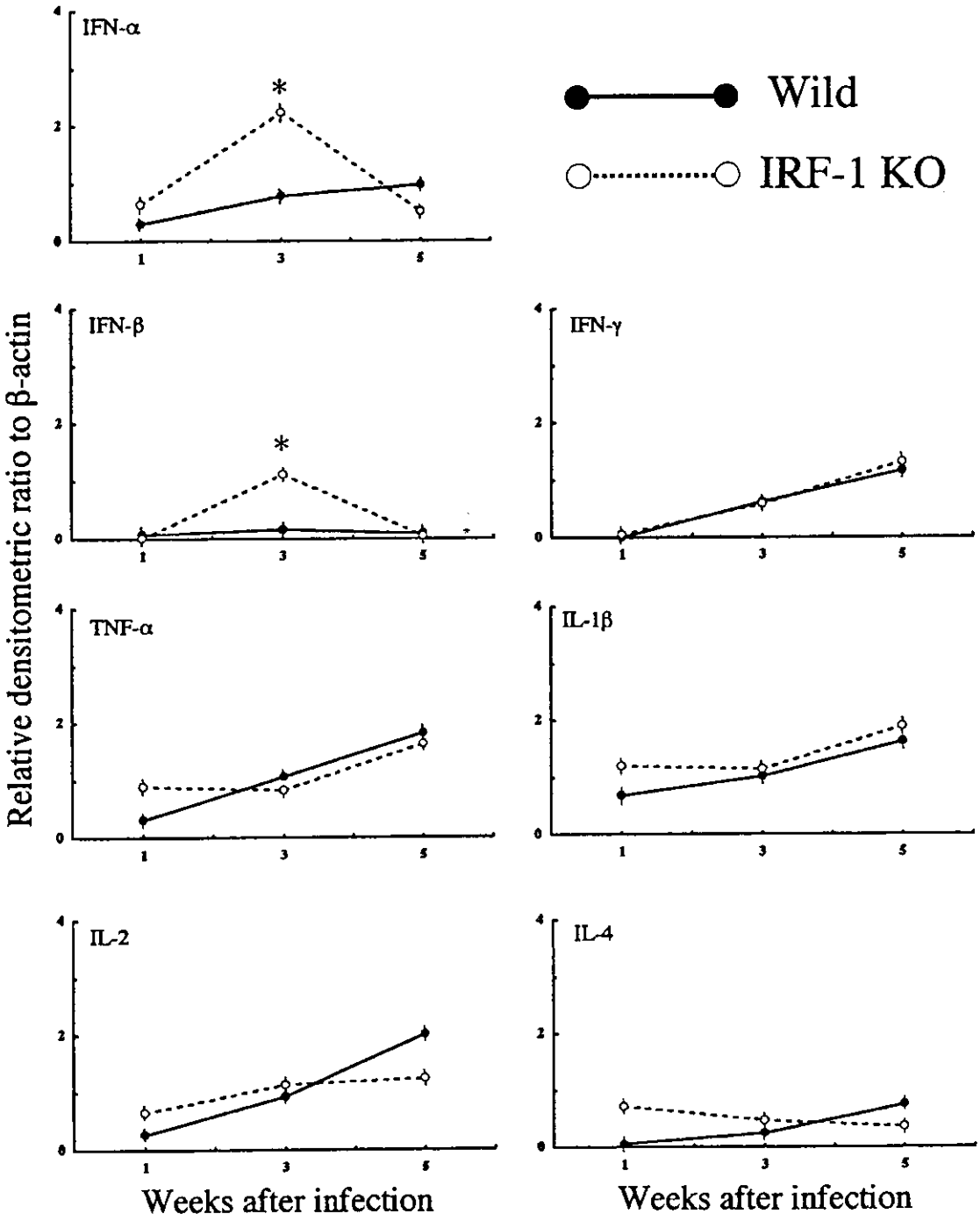

Fig. 5.

ical findings obtained from IRF-1 KO and WT mice showed similar changes three weeks after infection, but at five weeks, IRF-1 KO mice showed multifocal necrotic lesions in the lung, liver and spleen tissues (Fig. 3). Each necrotic lesion was characterized by central necrosis with massive accumulation of tubercle bacilli. These severe histopathological changes were not observed in the WT mice.

Electron microscopy demonstrated that alveolar macrophages in IRF-1 KO mice phagocytosed more tubercle bacilli, and that the engulfed tubercle bacilli were located in phagosomes and appeared to escape the killing mechanism of the host cells. No escape of *M. tuberculosis* from phagosomes to cytoplasm was

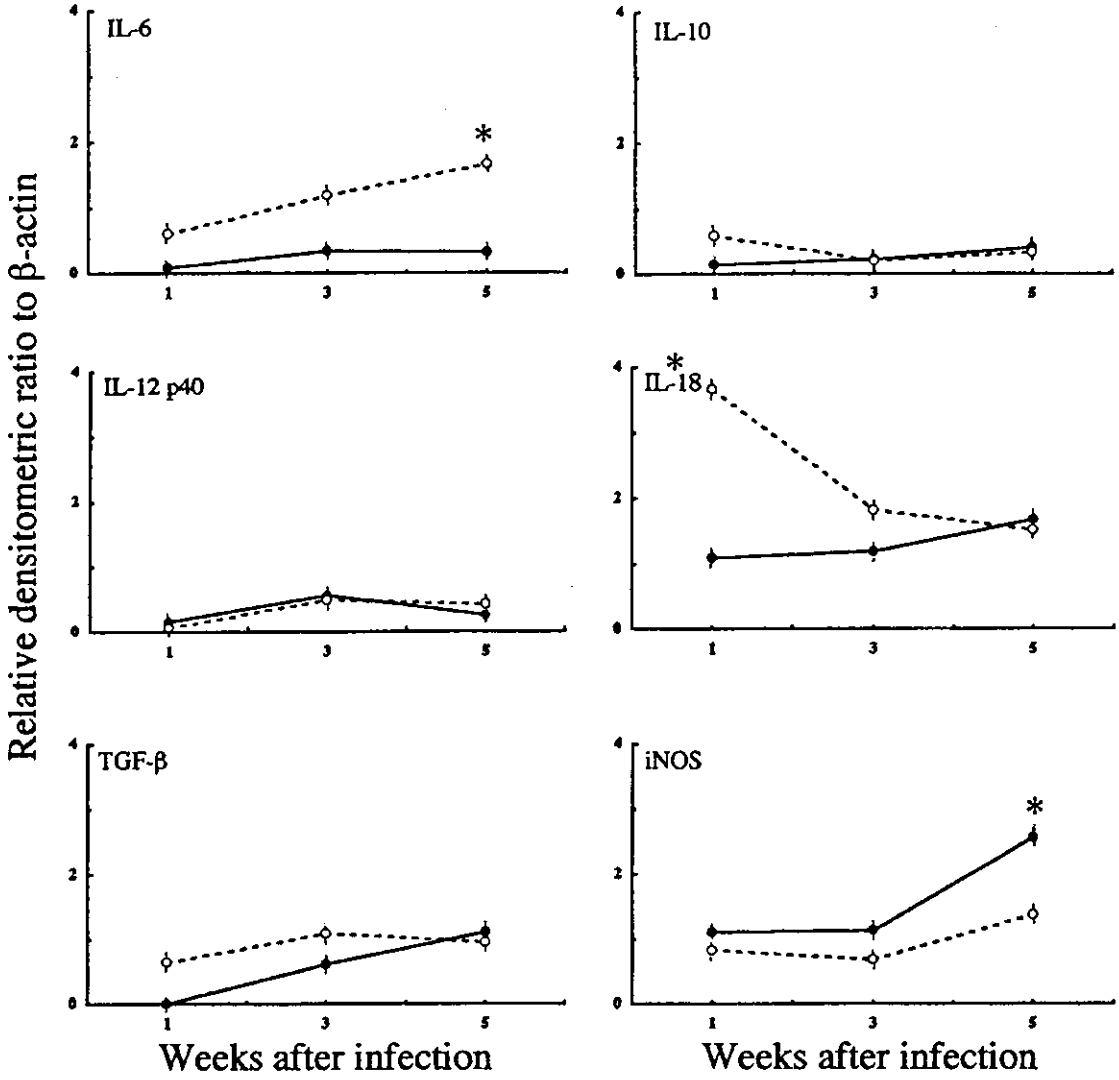

Fig. 5. Densitometric analysis of *in vivo* expression of various cytokines and iNOS mRNA in Kurono strain-infected mice by RT-PCR. The lung tissues (three mice each) of IRF-1 KO and WT mice were removed 1, 3 and 5 weeks after airborne infection. β-Actin gene primer sets were used as internal controls. The density of amplified bands was analyzed using NIH image software. * Indicates significant difference at $P<0.01$.

observed. Tubercle bacilli in the phagosomes of epithelioid macrophages contained many large vacuole-like structures (Fig. 4).

RT-PCR Analysis

Figure 5 shows densitometric analysis data of the RT-PCR in the infected lung tissues at 1, 3 and 5 weeks after aerial infection (three mice each). In IRF-1 KO mice, expression of IFN-γ and TNF-α mRNA was similar, but that of IFN-α and IFN-β mRNA was high until the third week after infection compared to that of WT mice. IFN-α mRNA expression was especially low at five weeks after infection. Expression of iNOS mRNA was depressed significantly in IRF-1 KO mice compared to that of WT mice ($P<0.01$). However, IL-18 and IL-6 mRNA expression was significantly higher in IRF-1 KO mice than in WT mice ($P<0.01$).

Discussion

We investigated the role of IRF-1 in mycobacterial infection *in vivo*. In the absence of IRF-1 activation, the IRF-1 KO mice were highly susceptible to *M. tuberculosis* infection. Under these conditions, the level of iNOS mRNA expression was significantly lower in IRF-1 KO mice than in the C57BL/6 WT mice. The NO activity of the alveolar macrophages from IRF-1 KO mice was significantly lower than that from WT mice, as

assessed by an NO assay with Griess reagent (6) (data not shown). The low NO activity may be due to reduced iNOS mRNA expression in the absence of IRF-1. Interestingly, IFN-α and IFN-β mRNA levels were high until the third week after infection in these IRF-1 KO mice, but IFN-γ mRNA expression was not changed. We expected low expression of IFN-α/β mRNA in our infection experiments. This high expression of IFN-α/β mRNA in mycobacterial infection may be explained as follows: IRF-3 and IRF-7 as well as IRF-1 activate IFN-α and IFN-β (11, 20). IRF-1 is deficient in our IRF-1 KO mice, but IRF-3 and IRF-7 are intact. Although IRF-1 is required for the induction of NO synthase in macrophages that are stimulated with IFN-γ, IRF-1 may activate type I and type II interferon promoters. We and other researchers have reported that IFN-γ KO mice were highly susceptible to *M. tuberculosis* (2, 4, 21). However, control by IFN-γ does not depend completely on the expression of the iNOS gene because a low dose of bacteria introduced directly into the lungs of mice that lack the iNOS gene is controlled almost as well as in intact mice (3). On the contrary, iNOS plays an essential role in IRF-1 KO mice infected with *M. tuberculosis* because these KO mice died of exacerbated mycobacterial infection.

It has been reported that IFN-α mRNA levels are high in mice infected with the highly virulent *M. tuberculosis* clinical isolate HN878, and that intranasal treatment with purified IFN-α/β results in higher lung bacillary load than no treatment and reduces survival even further (17). In our experiments, IFN-α/β mRNA levels were higher until the third week after infection than those of WT mice and increased IFN-α/β may exacerbate mycobacterial infection (1, 17). It has been reported that an IFN-stimulated response element binds IFN-stimulated gene factor 3 (ISGF3) that consists of p48, Stat (signal transducers and activators of transcription) 1, and Stat 2 (15, 26). In mice that are deficient in p48 and IRF-1, ISGF3 plays an essential role in both type I and type II IFN responses. Thus, the roles of IFN-α/β in mycobacterial infection have to be reconsidered by using p48- and ISGF3-deficient mice infected with *M. tuberculosis*. Not only iNOS, but also NK cells may play a major role for defense against mycobacterial infection. It is reported that IRF-1 KO mice show defective development of NK cells (19, 25). NK cells produce IFN-γ that activate alveolar macrophages and activated macrophages produce NO to kill tubercle bacilli.

TNF-α, IL-12, IFN-γ and IL-1β mRNA levels were not significantly changed in the IRF-1 KO mice compared to those in the C57BL/6 WT mice. Although it has been shown that the IRF-1 gene is induced by treatment with TNF-α and IL-1, the similar expression is not due to low IRF-1 induction (5). IRF-1 is required in iNOS induction in macrophages (13). The IRF-1 gene is also induced by IL-6 (5), but the levels of Th2 cytokine and IL-6 mRNA expression were high. It is thought that IL-6 is produced by macrophages and Th2, independent of the IRF-1-mediated pathway in our experiments. It is known that IL-10 inhibits IL-6 production and expression of IL-6 mRNA post-transcriptionally in monocytic cell lines (24). In our present study, the level of IL-10 mRNA expression was low, and this may explain the high expression of IL-6 mRNA. We also expected low expression of IL-12 mRNA in *M. tuberculosis*-infected IRF-1 KO mice because IL-12 production by macrophages is impaired in IRF-1 KO mice (9, 16). As intact IRF-8 is also associated with IL-12 mRNA expression, this may explain the unchanged expression of IL-12 mRNA in infected IRF-1 KO mice (12).

The histopathology of the IRF-1 KO mice infected with *M. tuberculosis* showed multifocal necrotic lesions of the lung, liver and spleen tissues, and *M. tuberculosis* elimination was severely impaired. The severity of the necrotic lesions is comparable to that in IFN-γ KO, TNF-α KO, NF-IL6 KO and NF-κB KO mice (2, 4, 14, 21, 23, 27). As $CD8^+$ T cells are reduced in number and NK cell activity is impaired in the IRF-1 KO mice used (13, 18, data not shown), these deficiencies may contribute to the progression of murine tuberculosis because NK cells produce IFN-γ and $CD8^+$ T cells are responsible for the killing of *M. tuberculosis*. However, in the case of *M. bovis* BCG infection, extensive granulomatous lesions were observed in the skin, lungs, livers and spleens, but the lesions were not lethal (13).

In summary, we have shown that IRF-1,which activates endogenous IFN-α and IFN-β promoters, plays an important role in the initial phase of mycobacterial infection. Although it is tempting to say that low levels of NO generated by the macrophages of IRF-1 KO mice are responsible for the severe course of mycobacterial infection, other possibilities cannot be ruled out.

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今村賞受賞記念講演

結核菌感染におけるサイトカインの役割に関する研究

菅原　勇

要旨：結核菌感染初期に関与する種々のサイトカインの役割をマウス結核モデルを利用して追究した。その中で，IFN-γとTNF-αが感染防御に最も重要であった。この所見は，ヒト結核にも深く関与しており，たとえばIFN-γ受容体1欠損症患者やヒト型抗TNF-α中和抗体を投与された慢性リウマチやクローン病患者で結核が多発することから裏付けられる。結核感染早期の病態を免疫学的に詳細に研究することにより，新しい結核診断法や治療法の開発につながることが期待される。

キーワーズ：サイトカイン，エアロソル感染，結核菌，インターフェロン・ガンマ，腫瘍壊死因子・アルファ

1. Introduction

Tubercle bacilli that are transported aerially to alveoli are phagocytosed by alveolar macrophages and, sometimes, overt tuberculosis results. The inflammatory sequence in tuberculosis involves exudative inflammation, proliferative inflammation and, finally, productive inflammation. In a clinical setting such as an outpatients clinic, the clinician can recognize proliferative and productive inflammation. However, clinicians have difficulty in recognizing the pulmonary exudative lesions that are induced by *M. tuberculosis* because the patients lack the symptoms and signs of tuberculosis and are treated for non-specific pneumonia. At the time when a definitive diagnosis is possible, patients are in the proliferative or productive stage of tuberculosis. Therefore, from early diagnostic and therapeutic viewpoints, it is interesting to examine what is going on immunologically in the exudative stage of tuberculosis. Murine tuberculosis can be used to study the aspects of human tuberculosis, particularly the exudative stage (early-phase tuberculosis). Tuberculosis is an airborne, chronic infectious disease. Thus, it is necessary to establish an inhalation exposure system (IES) before investigating the exudative stage of murine tuberculosis immunologically and pathologically. This memorial lecture focuses first on the establishment of an inhalation exposure system and then on the roles of cytokines (IFN-γ and TNF-α) in murine tuberculosis, mainly using specific gene knockout (KO) mice.

2. Inhalation exposure system

Animal (mouse and guinea pig) pulmonary tuberculosis models have been established using an automated inhalation exposure system (IES) apparatus (Glas-Col Corp., USA, Model 099CA-4212). This system includes four steps—preheating, nebulization, cloud decay and decontamination. The optimal conditions for infection experiments with *M. tuberculosis* H37Rv and Kurono strains were as follows: 10^6 colony-forming units (CFU) of tubercle bacilli; preheating for 15 min; nebulization for 90 min; cloud decay for 15 min and decontamination for 5 min[1]. When 10^4 CFU *M. tuberculosis* H37Rv strain was introduced into the lungs of interferon (IFN)-γ knockout mice using this IES apparatus and the mice were followed up for nine months, primitive cavitary lesions were formed. This apparatus was also useful for inhalation exposure experiments in guinea pigs, and it can be used for animal inhalation experiments with allergens.

3. Roles of IFN-γ in murine tuberculosis

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