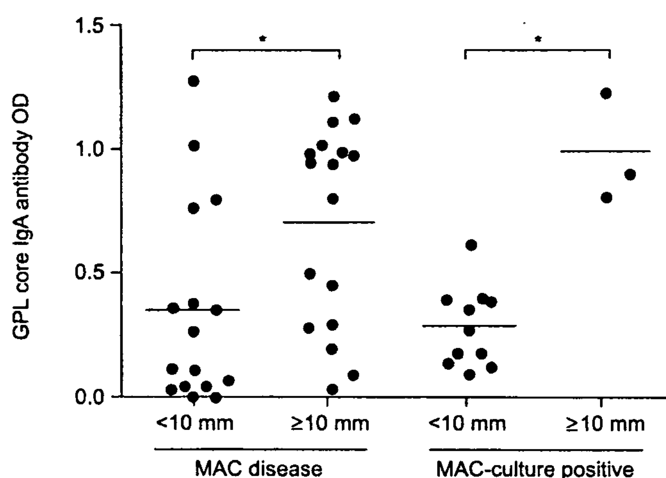

FIGURE 3. Serum level of glycopeptidolipid (GPL) core immunoglobulin (Ig)A antibody in patients who had nodular lesions <10 mm or ≥10 mm in diameter assessed by chest computed tomography in the *Mycobacterium avium* complex (MAC) disease group and in the MAC-culture positive group. The mean of each group is indicated by a horizontal bar. The levels of GPL core IgA antibody were significantly elevated in patients who had nodular lesions (≥10 mm) compared with patients who had small nodular lesions (<10 mm) in both the MAC disease group ($p<0.05$) and in the MAC-culture positive group ($p<0.05$), and the total of them ($p<0.001$). OD: optical density. *: $p<0.05$.

tolerance to the medication. However, 12 out of 33 patients with MAC disease and nine out of 14 MAC-culture positive patients did not undergo multidrug chemotherapy, including clarithromycin, following the present study. This was because most of these patients were >70 yrs old and/or did not have substantial symptoms and/or advanced or progressive radiographic abnormalities. Furthermore, treatment for MAC lung disease is expensive and is not covered by healthcare insurance in Japan. MAC lung disease is also difficult to treat and recurrence frequently occurs in MAC disease patients, even after completing multidrug chemotherapy, including clarithromycin. Many cases of recurrence have been experienced by the present authors, with the smear or culture test being positive over the 12 months following sputum-negative conversion during chemotherapy. This is because the radiographic active lesions, which are bronchiectasis or a cavity, have usually remained at the time of the sputum-negative conversion [8]. Thus, rapid diagnosis and treatment are required at an early stage before the completion of bronchiectasis or cavity lesions.

The serodiagnostic test used in the present study to detect serum GPL core antibodies could add useful information as a supplementary diagnostic aid [4, 16] and the present authors believe that this test may have future diagnostic applications. However, to include this serodiagnostic test in routine clinical practice, a study addressing the correlation between the antibody levels and radiographic findings was needed; the present study fulfils this requirement. The positive rates of the serological test were 71.4% for IgG, 100% for IgA and 84.6% for IgM in the MAC-culture positive group. If this serological test is combined with the ATS criteria, a better sensitivity to diagnose MAC lung disease without lung biopsy might be obtained.

The levels of GPL core antibody were similar in the MAC disease group and the MAC-culture positive group. Fifteen out of 33 (45.5%) of the MAC disease patients had received combination chemotherapy recommended by the ATS guidelines [7]. It is possible that this might have affected their antibody levels. However, the effects of treatment might be limited because they had a positive culture of MAC at enrolment, which meant the chemotherapy was not successful in converting the culture result from positive to negative at the time of serum sample collection. In the present authors' previous study, unsuccessful chemotherapy did not affect the level of GPL core antibody [4].

The level of IgA, but not IgG or IgM, GPL core antibody was significantly associated with the radiographic findings of the disease, but the reasons for this remain unclear. IgA is the predominant immunoglobulin isotype in mucosal tissue and is believed to be involved in the defence against viral and bacterial infection at this site. There are some published reports that are consistent with the present findings. RODRIGUEZ *et al.* [17] reported that IgA may play an important role in protection against mycobacterial infection in the respiratory tract by blocking the pathogens' entrance and/or by modulation of pro-inflammatory responses. In the present authors' previous study [4], the best serodiagnostic results for sensitivity and specificity for diagnosing MAC lung disease were obtained by measuring IgA. Moreover, WATANABE *et al.* [18] reported that total serum IgA was significantly higher in patients with MAC compared with those with pulmonary tuberculosis. These reports indicate that IgA antibody might play an important role in the chronic inflammation of mucous membrane of the respiratory tract in patients with MAC lung disease. The role of GPL core IgA antibody in protection against MAC is not clear and further studies are needed to address this question.

In summary, the present article documents that the level of immunoglobulin A glycopeptidolipid core antibody was significantly associated with radiographic findings. This observation should encourage the use of the serodiagnostic tests for *Mycobacterium avium* complex lung disease in clinical practice.

ACKNOWLEDGEMENTS
The authors wish to thank K. Maekura, Y. Yamamoto and M. Kobayashi for measurement of antibody levels.

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Kekkaku Vol. 82, No. 8 : 635-640, 2007

HIV感染症における結核感染診断に対しての QuantiFERON®-TB第2世代の有用性についての検討

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要旨：〔目的〕リンパ球のIFN-γ産生能を測定することによって結核感染の診断を行う方法（QFT-2G）が開発された。細胞性免疫機能が著しく低下するHIV感染症では，判定不可例の増加，感度の低下等が予想されるので，HIV感染症例におけるQFT-2Gの有用性について検討した。〔対象と方法〕①HIV感染症合併結核例，②抗HIV療法を受けているHIV感染症例，の2群につき，QFT-2G，CD4数，ツ反応等を検討した。〔結果〕①HIV感染症合併結核例13例では，QFT-2Gの感度は76.9%でツ反応の感度：発赤38.5%（硬結15.4%）に比べ有意に高かった。判定不可例が1例ありCD4数は16/μlと最も低い症例であった。②抗HIV療法施行中のHIV感染者25例にQFT-2Gを行い，判定不可例はなかった。CD4数は100～1157/μlであり，非結核既往群ではQFT-2G陽性はなく，結核既往群では陽性は3例（27.3%）であった。〔結論〕HIV感染症においてQFT-2GはCD4数の著減例では判定不可となる可能性があった。HIV感染症合併結核におけるQFT-2Gの感度は高く，十分有用であると考えられた。結核既往者の中にQFT-2G陽性者がおり，結核の再燃が起こるのか注意深い観察が必要である。
キーワーズ：結核，HIV感染症，QuantiFERON-TB第2世代，ESAT-6，CFP-10

はじめに

従来，結核感染の診断はツベルクリン反応（ツ反応）によって行われてきた。この方法はBCG未接種者においては感度，特異度ともに高く基本的には優れた方法であるが，BCG接種者においては，現れる反応が過去のBCG接種によるものか，最近受けた結核感染によるものかが区別できないという大きな問題がある。BCG接種に積極的に取り組んできたわが国では，結核感染の有無をツ反応で判定するのはしばしば困難を極める。そこにBCG接種の影響を受けない新しい結核診断法が開発された。特異的抗原刺激に対するリンパ球のインターフェロンγ（IFN-γ）産生能を測定することによって結核感染の診断を行う方法（QuantiFERON®-TB第2世代，以下QFT-2G）である。

QFT-2Gは，結核菌由来の特異抗原early secreted antigenic target 6（ESAT-6）とculture filtrate protein 10（CFP-10）の刺激による末梢血リンパ球のIFN-γ産生能を測定する検査法で，結核感染の診断有用性は高い。Moriら[1]によればQFT-2Gの結核感染の診断における特異度は98.1%，感度は89%である。

しかしながら，免疫抑制状態ではQFT-2Gの感度は低下する可能性があり，その有用性についての検討は乏しい。特に細胞性免疫機能が著しく低下するHIV感染症では，QFT-2Gの判定不可例の増加，結核を合併した場合のQFT-2Gの感度の低下が予想される。そこで，①結核発病時のHIV感染症例，②外来通院中のHIV感染症例（結核の既往例を含む）についてQFT-2Gを行い，

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(Received 22 Feb. 2007/Accepted 9 Apr. 2007)

QFT-2Gの判定不可例の頻度，結核発病時の感度等について検討した。

方　法

①対象は結核菌を確認できたHIV感染症合併結核のうち，結核の治療開始直前か，治療開始後1週間以内にQFT-2Gを行えた症例である。結核の既往歴がある症例およびすでに抗HIV薬の投与を受けている症例は除いた。対象症例は13例あり，男性12例，女性1例であった。年齢は20～67歳（中央値53歳）であった。

②対象は当院外来通院中で強力な抗HIV療法（highly active antiretroviral therapy: HAART）を行っていたHIV感染症例である。結核既往群と非結核既往群に分けて検討した。結核既往群は，結核診断時にHIV陽性と判明した症例で，当院で結核の治療を開始し，その後HAARTを開始した症例である。結核は治癒し，結核の治療を終了し外来にてHAART施行中に対象とした。非結核既往群は病歴上，結核の既往および結核患者との接触が明らかでない症例である。

外来通院中のHIV感染者のうちQFT-2Gを測定できた症例は25例あり，結核既往群11例（男女比9：2，29～58歳：中央値49歳），非結核既往群14例（男女比14：0，年齢31～65歳：中央値55歳）であった。

①，②の症例につきQFT-2G，CD4陽性Tリンパ球(CD4)数，ツ反応等について検討し，QFT-2Gの判定不可例の有無，QFT-2Gとツ反応との感度の比較等を行った。

ツ反応は発赤では発赤径10 mm以上を陽性とし，硬結ではATS/CDCの基準[2]によりHIV感染者の場合，硬結径5 mm以上を陽性とした。QFT-2Gの判定基準は次項に記載した。

QFT-2Gの感度とツ反応の感度の比較には，Fisher's exact probability testを用いた。

なお，QFT-2Gは2006年4月に保険収載となったが，それ以前の検査については当院倫理委員会の承認を得て，説明と同意のうえ行われた。

QFT-2G

Moriら[1]の方法に準じた。すなわち，被験者から静脈血をヘパリン加採血し，12時間以内にその一定量にESAT-6抗原，CFP-10抗原，陰性コントロールとしての生理的食塩水，陽性コントロールとしてのマイトジェン(phytohemagglutinin: PHA）を添加し，16～24時間37℃で培養した。培養後に上清を採取し，サンドイッチ酵素免疫測定法（ELISA法）でIFN-γの濃度を測定した。

刺激抗原ESAT-6，CFP-10により産生誘導されたIFN-γ値から陰性コントロールのIFN-γ産生値を差し引いた値のうち高値を選択した。0.35 IU/ml以上を陽性，0.1 IU/ml未満を陰性とした。その間の0.1以上0.35 IU/ml未満は判定保留とした。また，結核特異抗原によるIFN-γ産生値が0.35 IU/ml未満で，陽性コントロールから陰性コントロールを差し引いた値が0.5 IU/ml未満の場合は細胞性免疫応答が低下しているものとし，特異的免疫応答による測定値には信頼性がないとして，判定不可とした。

結　果

①QFT-2Gを行えたHIV感染症合併結核例13例の結核病変は，粟粒結核6例，肺結核6例，リンパ節結核1

Table 1 Results of QFT-2G in tuberculosis patients with HIV infection

Case	Gender/Age	CD4 counts (/μl)	Tuberculin skin test (mm) induration/erythema (double erythema)	QFT-2G
1. Miliary TB	M/45	16	0×0/3×3	Indeterminate
2. Miliary TB	M/60	23	0×0/0×0	Positive
3. Pulmonary TB	M/59	27	0×0/0×0	Intermediate
4. Pulmonary TB	M/57	36	0×0/0×0	Positive
5. Pulmonary TB	M/47	48	11×10/61×41	Positive
6. Miliary TB	M/53	60	0×0/0×0	Intermediate
7. Miliary TB	F/38	63	0×0/15×13	Positive
8. Pulmonary TB	M/66	68	0×0/0×0	Positive
9. Miliary TB	M/63	81	0×0/5×5	Positive
10. Pulmonary TB	M/36	101	0×0/0×0	Positive
11. Miliary TB	M/67	199	0×0/15×15	Positive
12. Lymph node TB	M/41	245	15×17/20×20 (40×57)	Positive
13. Pulmonary TB	M/20	320	0×0/16×21	Positive
	Median 53	Median 63	Sensitivity erythema 38.5% (induration 15.4%)	Sensitivity 76.9%

TB: tuberculosis

Table 2　Results of QFT-2G in HIV-infected patients under HAART

	Number	QFT-2G Positive	QFT-2G Intermediate	QFT-2G Negative	CD4 counts (/μl) Median (range)
Past history of TB (Completion of TB therapy)	11	3	2	6	348 (124–561)
No history of TB	14	0	2	12	496 (100–1157)

TB: tuberculosis

Table 3　Results of QFT-2G in HIV infected persons after completing of TB therapy

	QFT-2G	Gender/Age	Duration between QFT-2G and TB diagnosis (months)	CD4 counts (/μl) (at TB diagnosis)	Tuberculin skin test (induration: mm) (at TB diagnosis)
1.	Positive	F/40	19	218 (63)	20 (0)
2.	Positive	M/42	48	234 (11)	13 (0)
3.	Positive	F/29	67	396 (72)	25 (6)
4.	Intermediate	M/57	12	124 (27)	12 (0)
5.	Intermediate	M/58	93	367 (423)	8 (0)
6.	Negative	M/46	30	178 (2)	9 (0)
7.	Negative	M/55	42	320 (106)	16 (0)
8.	Negative	M/43	50	549 (188)	13 (31)
9.	Negative	M/53	55	348 (31)	8 (0)
10.	Negative	M/50	77	561 (111)	13 (0)
11.	Negative	M/49	85	518 (35)	0 (0)
	Positivity rate 27.3%	Median 49	Median 50	Median 348 (63)	Positivity rate 90.9 (18.2) %

TB: tuberculosis

例であった (Table 1)。CD4数は16～320/μl (中央値63/μl)であった。QFT-2Gの結果は, 陽性：13例中10例 (76.9%), 判定保留：13例中2例 (15.4%), 判定不可：13例中1例 (7.7%) であった。ツ反応の陽性率は発赤で判定した場合38.5%, 硬結で判定した場合15.4%であった。QFT-2Gの感度はツ反応硬結の感度よりも有意に高かった ($p<0.01$)。

判定不可例のCD4数は16/μlと最も低値であった。この症例はHAARTを開始後, CD4数が増加し, 陽性コントロールが認められるようになった。しかし, その時点のQFT-2Gは陰性であった。

② 外来通院中のHIV感染者のうちQFT-2Gを測定できた症例25例は, 全例にHAARTが施行され, CD4数は結核既往群124～561/μl (中央値348/μl), 非結核既往群100～1157/μl (中央値496/μl) であり, CD4数が著しく低下している例はなかった (Table 2)。いずれも陽性コントロールに対するIFN-γ産生は良好で, 判定不可例はなかった。非結核既往群14例ではQFT-2G陽性はなく, 判定保留2例, 陰性12例であった。結核既往群11例 (Table 3) では, HAARTによりCD4数が増加 (中央値63→348/μl) し, ツ反応は硬結陽性率が18.2%から90.9%へ上昇した。しかし, QFT-2G陽性者は3例 (27.3%) であり, ツ反応の陽性率に比べ低かった。判定保留2例, 陰性6例であった。

考　察

われわれの対象症例数は少なかったが, HIV感染者においてもCD4数が著しく低下していなければQFT-2Gは判定不可とならず判定可能であると判明した。また, HIV感染者における活動性結核の発病時においては, QFT-2Gはツ反応に比べ陽性率がより高く, 結核感染診断の有用性がきわめて高いと考えられた。

結核感染の診断はツ反応で行われてきたが, ツ反応の反応性は細胞性免疫機能に左右される。活動性結核であっても細胞性免疫機能が低下した状態ではツ反応の陽性率は低下し, 偽陰性例が増加する。特に細胞性免疫機能が著しく低下するHIV感染症では, ツ反応の診断能力は低下する。Johnsonら[3]によれば活動性結核を合併したHIV感染者の30%, AIDS発病例の60%以上はツ反応硬結径が10 mm以下であったという。したがって, HIV感染症では結核感染の診断法としてツ反応の有用性は低下する。そこで, 近年開発され結核感染の診断において高い感度を示すIFN-γ産生能を測定する方法が期待される。

IFN-γ測定法を用いてHIV感染症における結核診断を行う際に注意が必要な点は, 細胞性免疫機能が低下しているので, PHAによる陽性コントロールが確実に得られるかという点である。これが得られない場合は判定

不可となる。当院の症例では結核合併例における判定不可例が1例（7.7%）あり，この症例は13例中CD4数が最も低値（16/μl）であった。やはり免疫機能が著しく低下した症例は判定不可となる可能性があるので，この点については認識しておくべきである。しかし，外来通院中のHAART施行例25例におけるQFT-2G検査では，判定不可例はなかった。25例のCD4数値は100～1157/μl（中央値396/μl）であり，CD4数が著しく低下していなければQFT-2Gは判定不可にならないと考えられた。

Brockら[4]によれば590名のHIV感染者にQuanti-FERON-TB In-Tube test（In-Tube version）を行ったところ，陽性者には潜在性結核感染症のリスクをもった症例や結核の既往のある症例が多かった。しかし，CD4数が少ないほど判定不可例が多かった。

QFT-2Gと同様にESAT-6およびCFP-10の両特異抗原を利用してリンパ球を刺激し，反応性のIFN-γの産生を測定するT-SPOT.TBという方法がある。これはIFN-γ産生測定法としてenzyme-linked immunospot（ELISPOT）assayを用いる方法である。T-SPOT.TBについてはHIV感染症においても十分にPHAに反応し，CD4数に影響を受けないという報告[5]がある。QFT-2GとT-SPOT.TBを比較した報告[6]では，判定不可例はQFT-2G：11%，T-SPOT.TB：3%とQFT-2Gのほうが多かった。特に5歳以下ではQFT-2Gでは判定不可が多かった。南アフリカのHIV感染症も結核も非常に多い地域での活動性結核を発病していない160名（HIV陽性者74名，陰性者86名）に対して，T-SPOT.TB，QFT-2G，ツ反応を施行した報告[7]がある。HIV陽性者は陰性者に比べ，ツ反応の陽性率は有意に低かった。しかし，T-SPOT.TB（陽性率：HIV陽性者52%，HIV陰性者59%）もQFT-2G（陽性率：HIV陽性者43%，HIV陰性者46%）も陽性率にHIV陽性・陰性に差がなかった。両者とも中等度に進んだHIV感染症では感度が落ちないとしている。ただし，判定不可例はELISPOTで1%，QFT-2Gで7%認められている。上記の報告[6][7]をみるとT-SPOT.TBに比べ，QFT-2Gは免疫能が低下した状態では判定不可例が生じやすい可能性があるので注意が必要である。

当院のAIDS合併結核におけるQFT-2Gの結核感染診断の感度は76.9%であり，ツ反応に比べ有意に高率であり，HIV感染症においても結核感染の診断には有用な検査法と考えられた。また，判定保留症例が2例あったが，いずれもCD4数が27/μl，60/μlと低値であることを考慮すると，この2例においても結核感染を示している可能性が高い。免疫低下状態における判定保留症例の扱いについてはさらに症例を集め検討すべきである。

HIV感染症合併結核におけるIFN-γ産生能測定法の有用性についての報告は少なく，ELISPOTについての報告が散見されるのみである。Chapmanら[8]は39例のHIV感染症合併結核おけるELISPOTの感度は90%と非常に高く，有用であったと報告している。Liebeschuetzら[9]によるAfricaの小児の前向き研究では，ELISPOTの結核診断の感度は83%であり，ツ反応の感度の63%に比較し有意に高かった。免疫機能が低下していると考えられる3歳以下の小児，HIV感染症，低栄養状態における結核感染に対するツ反応の感度は51%，36%，44%であった。これに対してELISPOTはそれぞれ，85%，73%，78%と高値であり，ELISPOTは免疫機能の低下にも影響を受けにくいという結果であった。この2報告はELISPOTについてであるが，当院のHIV感染症合併結核におけるQFT-2Gの感度76.9%はほぼ同等の結果であった。今後さらに症例を増やし検討したい。

ELISPOTはHIV感染者においても判定不可例が少なく期待される検査法であるが，QFT-2Gに比べ検査法が煩雑であり，現時点では容易にわが国で利用できる状況にはない。QFT-2GはHIV感染者においてもツ反応に比べより有用な結核感染診断法であることは明らかであり，その特徴を十分理解して適切に用いるべきである。

今回の検討で，HAART施行中の結核既往群では，HAARTによりCD4数が増加し細胞性免疫が回復し，ツ反応が90.9%と高率に陽転化していたが，QFT-2G陽性者は27.3%と少なかった。結核の治療歴がある非HIV感染者におけるQFT-2G陽性率については，当院で行った検討[10]では，結核の治療終了後1年以上経過している患者43例中，QFT-2G陽性20例（46.5%），判定保留9例（20.9%），陰性14例（32.6%）であった。非HIV感染者に比べ，HIV感染者では結核の治療終了後のQFT-2G陽性率はやや低い傾向があった。結核の治療終了後もQFT-2Gが陽性であることの意味付けは難しく，依然として結核菌が存在することを示すのか，免疫の記憶だけが残っているのか議論の多いところである。QFT-2G陽性者についてはQFT-2Gの変動，結核再燃の有無などについて経過を注意深く追う必要がある。

また，HIV感染者における接触者検診や潜在結核感染症の診断にQFT-2Gは有用と考えられる[9]ので，今後のデータの蓄積が必要である。

結　論

（1）HIV感染症合併結核例の13例中1例にQFT-2G判定不可例を認めた。CD4数が著しく低下した症例では判定不可となる可能性がある。

（2）HIV感染症合併結核におけるQFT-2Gの感度は76.9%であり，ツ反応の15.4%よりも有意に高かった。HIV感染症合併結核においてもQFT-2Gは結核感染診断に十分有用であると考えられた。

（3）結核治療終了例のうち QFT-2G陽性者が27.3%あり，この中から結核の再燃が起こるのか注意深い観察が必要と思われた。

〔付記〕

この研究の一部は平成18年度厚生労働科学研究費補助金エイズ対策研究事業『重篤な日和見感染症の早期発見と最適治療に関する研究/主任研究者：安岡　彰（長崎大学医学部歯学部附属病院感染制御教育センター）』の分担研究として行われた。

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Original Article

USEFULNESS OF A WHOLE BLOOD INTERFERON GAMMA ASSAY (QuantiFERON®-TB-2G) FOR DETECTING TUBERCULOSIS INFECTION IN HIV-INFECTED PERSONS

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Abstract [Background] New blood test (QuantiFERON®-TB-2G: QFT-2G), based on detection of IFN-gamma released by T cells in response to *M. tuberculosis* specific antigens, has the high sensitivity and specificity for diagnosis of tuberculosis. However, it is essential to evaluate this T cell-based approach in individuals with HIV-associated impairment in T cell immunity.

[Methods] We assessed the usefulness of QFT-2G on diagnosis of tuberculosis in 13 HIV-infected patients with tuberculosis and the performance of 25 HIV infected persons under highly active antiretroviral treatment (HAART). QFT-2G, CD4 counts, and tuberculosis skin test and so on were examined.

[Results] The sensitivity of QFT-2G in HIV-infected patients with tuberculosis was 76.9%, which was significantly higher compared with tuberculin skin test, 15.4%. There was one indeterminate case of which CD4 count was $16/\mu l$, the lowest count among the all patients. CD4 counts of 25 HIV infected persons under HAART were between 100 and $1157/\mu l$. There were 3 QFT-2G positive cases among them, who had past history of tuberculosis.

[Conclusion] Although the very low CD4 counts in HIV-infected patients might adversely affect QFT-2G performance, the sensitivity of QFT-2G in the most of HIV-infected patients with tuberculosis was high, and it was thought that it was useful enough to diagnose tuberculosis infection. Careful observation is required in whether the recurrence of tuberculosis takes place among QFT-2G positive persons who have past history of tuberculosis.

Key words: Tuberculosis, HIV infection, QuantiFERON-TB-2G, ESAT-6, CFP-10

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MAJOR ARTICLE

Diagnosis of Active Tuberculous Serositis by Antigen-Specific Interferon-γ Response of Cavity Fluid Cells

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Background. To develop a more accurate methodology for diagnosing active tuberculous pleurisy, as well as peritonitis and pericardits of tuberculous origin, we established an antigen-specific interferon γ (IFN-γ)–based assay that uses cavity fluid specimens.

Methods. Over a 19-month period, 155 consecutive, nonselected patients with any cavity effusion were evaluated. Study subjects were 28 patients with bacteriologically confirmed active tuberculous serositis and 47 patients with definitive nontuberculous etiology. Culture was performed for 18 h with fluid mononuclear cells in the supernatant of the effusion together with saline or *Mycobacterium tuberculosis*–specific antigenic peptides, early secretory antigenic target 6 and culture filtrate protein 10. IFN-γ concentrations in the culture supernatants were measured.

Results. In patients with active tuberculous serositis, antigen-specific IFN-γ responses of cavity fluid samples were significantly higher than those of nontuberculous effusion samples. Area under the receiver operating characteristic (AUROC) curve was significantly greater for cavity fluid IFN-γ response (AUROC curve, 0.996) than for cavity fluid adenosine deaminase and whole-blood IFN-γ responses (AUROC curve, 0.882 and 0.719, respectively; $P = .037$ and $P < .001$, respectively). Although the AUROC curve was greater for cavity fluid IFN-γ response than for background cavity fluid IFN-γ level (AUROC curve, 0.975), the AUROC curves were not statistically significantly different ($P = .74$). However, multivariate logistic regression analysis revealed that cavity fluid IFN-γ responses were significantly associated with the diagnosis, even after adjustment for background IFN-γ level (adjusted odds ratio, 1.21; 95% confidence interval, 1.03–1.42; $P < .001$).

Conclusions. The cavity fluid IFN-γ assay could be a method for accurately and promptly diagnosing active tuberculous serositis.

Tuberculosis is a serious infectious disease that threatens the health of mankind, even in the modern world [1]. Active tuberculous pleuritis, peritonitis, and pericarditis should always be considered as possible causes of cavity fluid in the daily practice of clinical medicine. Their definitive diagnosis is based on the identification of *Mycobacterium tuberculosis* after culture of effusion or tissue biopsy specimens. However, the rate of positive results of such cultures of pleural fluid specimens is not high (<30%) [2]. In addition, because bacteriological results cannot be obtained rapidly, antituberculous agents should, in some cases, be instituted on the basis of clinical speculation without waiting for the definitive diagnosis. Measurement of nonspecific markers, such as adenosine deaminase (ADA) and IFN-γ, in the supernatant of fluid specimens has been used to diagnose pleural tuberculosis [3, 4]. A meta-analysis concluded that maximum joint sensitivity and specificity was 93% for the ADA assay and 96% for the IFN-γ assay [5]. It has been reported that the levels of these markers are also increased in the context of other diseases [6–9], and there is some contention as to the use of these tests [10, 11]. Thus, it would be desirable to develop a more specific diagnostic method for active tuberculous serositis (ATBS).

Active tuberculous pleuritis is thought to be caused by the spread of a small subpleural or hilar lymph node

Received 1 May 2007; accepted 2 August 2007; electronically published 8 November 2007.

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Clinical Infectious Diseases 2007;45:1559–67

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1058-4838/2007/4512-0005$15.00
DOI: 10.1086/523591

caseous focus of *M. tuberculosis* into the cavity, and T cells that specifically respond to tuberculous antigens and produce IFN-γ are recruited in the pleural cavity of patients with active tuberculous pleuritis [12, 13]. *M. tuberculosis*–specific protein antigens early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are absent from the genomes of all bacille Calmette-Guérin substrains and most nontuberculous mycobacteria, have been identified [14]. A specific method for the detection of tuberculous infection has been developed in which IFN-γ production (the number of IFN-γ–producing cells) is measured after stimulation of peripheral blood with these antigens in vitro [15–19]. We hypothesized that, when cavity fluid mononuclear cells of patients with ATBS were stimulated with the antigenic peptides ESAT-6 or CFP-10, IFN-γ responses that are greater than those achieved using the stimulation of peripheral blood might be expected. We report our results, which suggest the possibility of accurate diagnostic procedures for ATBS that reflect the local immune response in the cavity.

PATIENTS AND METHODS

Patients and evaluation of their cavity effusion specimens. Over a 19-month period from November 2004 through June 2006, 155 consecutive, nonselected patients with any cavity effusion were evaluated at the inpatient department of Tokyo National Hospital (Tokyo, Japan). Either cavity centesis or endoscopic procedures, including thoracoscopic and perioto-neoscopic procedures, was performed for all patients; 75 patients for whom the etiology of cavity effusion could be definitively identified were enrolled. Table 1 shows the profiles and the clinical diagnoses that these patients received. All patients with tuberculosis had received <7 days of antituberculous chemotherapy at the time of testing. None of these patients had conditions, such as HIV infection, associated with severe immunocompromise, and none were receiving immunosuppressive drugs.

The differential diagnosis based on the evaluation of the pleural effusion specimens was made using the following criteria. A definitive diagnosis of active tuberculous pleuritis, peritonitis, or pericarditis was made on the basis of positive culture results and identification of *M. tuberculosis* in cavity effusion or parietal tissue specimens. With regard to nontuberculous pleuritis, peritonitis, and pericarditis, the diagnosis of neoplastic pleuritis, peritonitis, or pericarditis was based on detection of malignant cells in pleural effusion or tissue specimens; parapneumonic effusion was defined by the presence of fever, spu-

Table 1. Profiles of patients with cavity effusion.

Variable	Patients with active tuberculous serositis (n = 28)	Patients with nontuberculous effusion (n = 47)	All (n = 75)
Age, years			
Mean ± SD	60.5 ± 22	72.1 ± 10.8	67.9 ± 17.1
Range	20–91	46–91	20–91
Sex			
Male	24 (86)	40 (85)	65 (87)
Female	4 (14)	7 (15)	10 (13)
Clinical diagnosis			
Active tuberculous pleuritis	26	0	...
Active tuberculous peritonitis	1	0	...
Active tuberculous pericarditis	1	0	...
Nontuberculous pleuritis	0	45	...
Neoplastic pleuritis	0	24	...
Lung cancer	0	22	...
Malignant mesothelioma	0	2	...
Parapneumonic effusion	0	12	...
Heart failure	0	5	...
Asbestos pleural effusion	0	1	...
Posttraumatic pleural effusion	0	1	...
Mycobacterium avium pleuritis	0	1	...
Hypothyroidism (pleural effusion)	0	1	...
Cancerous peritonitis	0	1	...
Cancerous pericarditis	0	1	...

NOTE. Data are no. or no. (%) of patients, unless otherwise indicated.

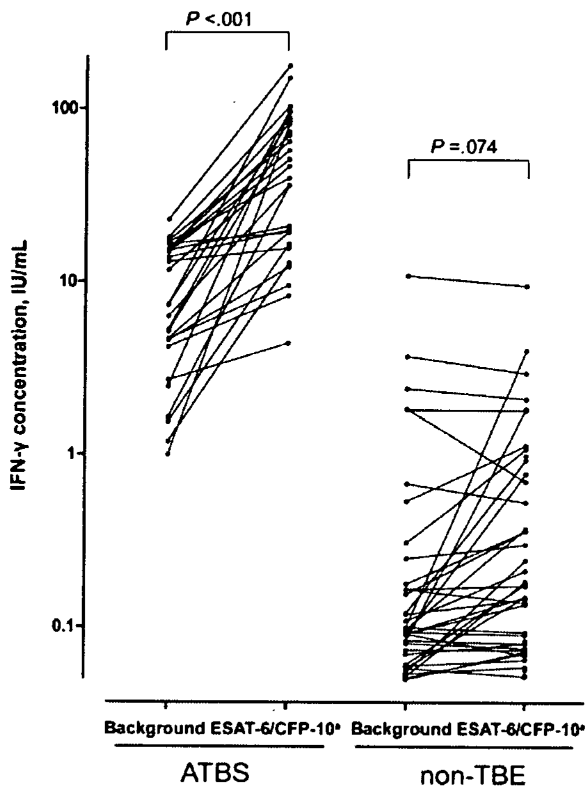

Figure 1. IFN-γ production in response to stimulation with *Mycobacterium tuberculosis*-specific antigen. Pleural, peritoneal, and pericardial fluid cells obtained from patients with active tuberculous serositis (ATBS) or nontuberculous effusion (non-TBE) were harvested and suspended in the individual supernatant of the effusion at 1–5 million cells/mL, stimulated with saline or *M. tuberculosis*-specific antigens, and incubated for 18 h, after which IFN-γ concentrations in the culture supernatant were measured by ELISA. If, in an initial assay, a supernatant was found to contain >15 IU/mL of IFN-γ, the specimen was diluted with zero standard and reassayed. IFN-γ concentrations in each patient, which were expressed as background saline control IFN-γ concentrations and concentrations after stimulation with specific antigens (early secretory antigenic target 6 [ESAT-6] and culture filtrate protein 10 [CFP-10]), are shown as closed circles connected with lines. Student's *t* test was used for differences between the ATBS and non-TBE groups. IFN-γ values after antigen stimulation are the measured values, not values subtracted from the corresponding background control values. ªThe higher IFN-γ concentrations after stimulation with either antigenic peptides ESAT-6 or CFP-10 are represented.

tum, infiltrates on chest radiograph, an infectious inflammatory response detected by serological testing, and improvement after the administration of antibiotics. Heart failure was assigned as the cause of pleural effusion if cardiac dysfunction was evident on ultrasonographic cardiograph and improvement was noted after the administration of diuretics or cardiotonic drugs. Asbestos pleural effusion was diagnosed on the basis of detection of asbestos particles or fibrous patches in pleural biopsy specimens. Posttraumatic hemothorax was indicated when there was evident pooling of blood in the thorax after chest trauma. Pleuritis due to *Mycobacterium avium* was considered on the basis of identification of *M. avium* in pleural effusion specimens.

Stimulation of cavity fluid cells with* M. tuberculosis*–specific antigens (cavity fluid IFN-γ assay). Twenty to 50 mL of cavity effusion were centrifuged at 2000 rpm for 15 min. The supernatant was separated, and the number of nucleated cells in the cell compartment was determined. Cells were then suspended at 1–5 million cells/mL in the individual supernatant of the effusion. One milliliter of this cell suspension was then added to 4 wells of 24-well plates (NUNC) and underwent culture in the presence of *M. tuberculosis* antigen peptides ESAT-6 or CFP-10 at a final concentration of 1 μg/mL (QuantiFERON-TB 2G; Cellestis) [19]. To the remaining 2 wells, physiological saline was added instead of the antigens, because a measurement of background IFN-γ of cavity fluid and phytohemagglutinin (5 μg/mL) was used as a positive control. Cells were incubated at 37°C for 18 h in a 5% CO_2 incubator, and the cultured supernatant was harvested for measurement of IFN-γ concentration. The value of the cavity fluid assay was defined as the difference between the determined higher value after stimulation with either ESAT-6 or CFP-10 and the value of the measurement of background IFN-γ. The whole-blood IFN-γ assay was performed as previously described [19].

Measurement of IFN-γ and ADA concentrations. IFN-γ concentration was measured using the QuantiFERON-CMI ELISA (Cellestis) according to procedures described in the manufacturer's instructions. The IFN-γ values were expediently replaced with 0.05 when the difference between the antigen-stimulated level and the background level was negative or <0.05 IU/mL (lower limit of measurement). If, in an initial assay, the supernatant was found to contain >15 IU/mL of IFN-γ (upper limit of measurement), the specimen was diluted with zero standard and reassayed. When both stimulated and background IFN-γ concentrations were extremely high, we performed another culture using plasma samples from each patient for medium to decrease the background IFN-γ concentration. The result of the whole-blood assay was considered to be indeterminate if the value for the positive control sample was <0.5 IU/mL after subtraction of the value for the negative control sample. ADA concentration in the supernatant of cavity fluid samples was measured by AUTO A MIZUHO ADA (Mizuho Medy). Three technicians with expertise in laboratory work were blinded to the clinical information of the patients, results of other assays in this study, and the reference standard.

Statistical analysis. Univariate analyses were performed using Student's *t* test and the Mann-Whitney *U* test. We constructed a receiver operating characteristic (ROC) curve by

Table 2. IFN-γ response in cavity fluid and whole-blood cells to tuberculous-specific and nonspecific antigens and adenosine deaminase (ADA) levels in patients with active tuberculous serositis and patients with nontuberculous effusion.

Variables	Patients with active tuberculous serositis (n = 28)	Patients with nontuberculous effusion (n = 47)	P
Background IFN-γ level in cavity fluid	7.4 (4.5–15.2)	0.09 (0.05–0.16)	<.001
ESAT-6–background[a]	26.4 (6.8–49.4)	0.05 (0.05–0.08)	<.001
CFP-10–background[b]	12.2 (2.8–37.7)	0.05 (0.05–0.09)	<.001
ESAT-6 or CFP-10–background[c]	34.8 (7.3–74.3)	0.05 (0.05–0.19)	<.001
Mitogen–background[d]	22.6 (2.1–55.1)	6.90 (0.32–21.5)	.009
Whole-blood IFN-γ assay result[e]	0.54 (0.3–0.9)	0.07 (0.05–0.54)	<.001
ADA concentration[f]	61.3 (43.0–71.5)	13.80 (7.95–27.5)	<.001

NOTE. Data are median value (interquartile range). Mann-Whitney U test was used for differences in IFN-γ and ADA concentrations between the patients with active tuberculous serositis and patients with nontuberculous effusion. CFP-10, culture filtrate protein 10; ESAT-6, early secretory antigenic target 6.

[a] Difference between the determined IFN-γ concentration after stimulation with ESAT-6 and the background IFN-γ concentration in cavity fluid.

[b] Difference between the determined IFN-γ concentration after stimulation with CFP-10 and the background IFN-γ concentration in cavity fluid.

[c] Difference between the determined higher IFN-γ concentration after stimulation with either ESAT-6 or CFP-10 and the background IFN-γ concentration.

[d] Difference between the determined IFN-γ concentration after stimulation with mitogen and the background IFN-γ concentration in cavity fluid.

[e] None of the patients had indeterminate results, and 1 patient with tuberculosis was unavailable.

[f] Not performed for 1 patient with tuberculosis.

plotting the rate of sensitivity against the rate of false-positive results over a range of cutoff values of IFN-γ and ADA for the assessment of diagnostic accuracy. Area under the ROC curve was calculated using the trapezoidal rule. Standard errors of the areas and comparisons between assays were calculated using a method described elsewhere [20]. Pairwise comparisons were made between area under the ROC curve for the cavity fluid IFN-γ assay and that for each of the following measures: background IFN-γ and ADA assays and the whole-blood IFN-γ assay. Adjustment for multiple comparisons was made using the Dunn-Sidák method. To assess whether the cavity fluid assay lends additional diagnostic information to that provided by the background IFN-γ level, these 2 measurements were introduced as predictors in a multivariate logistic regression model. These 2 explanatory variables were treated as continuous variables. $P < .05$ was considered to be statistically significant. All statistical analyses were performed using Stata, version 9 (Stata).

This study was approved by the ethics review committee of our hospital. We obtained informed consent from all participants in the study.

RESULTS

The mean age of patients with nontuberculous effusion (non-TBE) was significantly higher than that of patients with ATBS (72.4 years vs. 60.5 years; $P = .013$). There was no statistically significant difference in sex between the groups (85% male vs. 81% male; $P = .745$) (table 1).

Figure 1 shows results of the cavity fluid IFN-γ assay for each subject (in both the ATBS and non-TBE groups), including background IFN-γ values and the highest values for either of the stimulation antigens, ESAT-6 or CFP-10. The IFN-γ concentration was significantly increased after antigen stimulation, compared with the background IFN-γ concentration, in every patient with ATBS ($P < .001$). However, in patients with non-TBE, IFN-γ concentrations did not significantly increase after stimulation with antigens ($P = .074$)

The median values and the interquartile range of background cavity fluid IFN-γ, antigen-specific cavity fluid IFN-γ, whole-blood, and ADA assays are shown in table 2. Individual values for these parameters, as well as the result of the cavity fluid assay multiplied by the background IFN-γ level, are indicated by dots in figure 2. Background IFN-γ concentrations were significantly higher in patients with ATBS than in patients with non-TBE. Cavity fluid IFN-γ responses to both ESAT-6 and CFP-10 were significantly greater for patients with ATBS than for patients with non-TBE. Likewise, when an IFN-γ response was represented by a higher value of response to either ESAT-6 or CFP-10, the difference between the values for the ATBS group and the non-TBE group was greater. IFN-γ responses to mitogen were significantly greater for patients with ATBS than

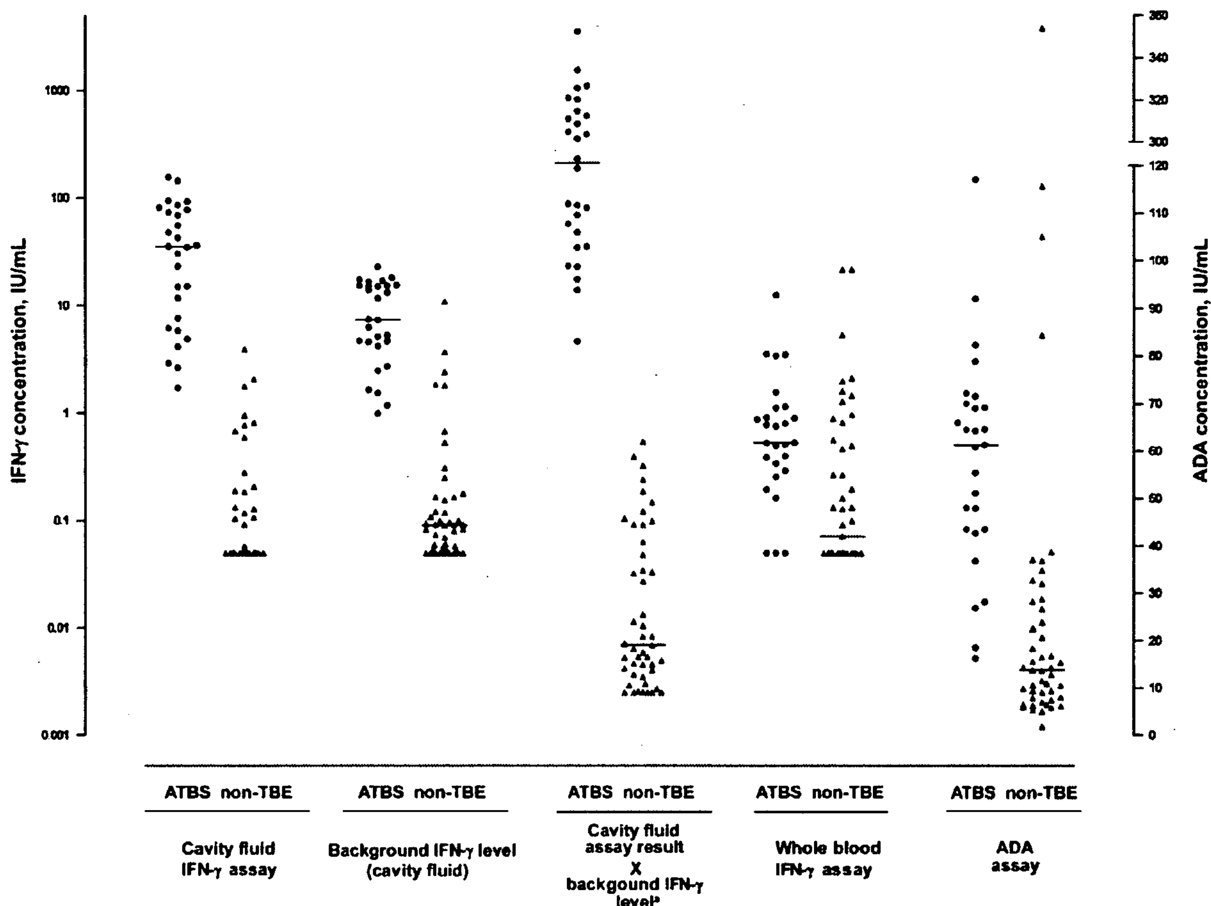

Figure 2. IFN-γ concentrations in cavity fluid and whole-blood and adenosine deaminase (ADA) concentrations in patients with active tuberculous serositis (ATBS) and in patients with nontuberculous effusion (non-TBE). Individual results of whole-blood IFN-γ, ADA, cavity fluid IFN-γ, and background control IFN-γ assays for patients with ATBS or non-TBE are shown. IFN-γ values are represented by the higher value of response to either early secretory antigenic target 6 (ESAT-6) or culture filtrate protein 10 (CFP-10) after subtraction of each background control IFN-γ value for the cavity fluid and whole-blood IFN-γ assays. Values for patients with ATBS and non-TBE are represented by closed circles and triangles, respectively. Horizontal lines in the columns represent the median value in each group. [a]Values calculated by multiplying the result of the cavity fluid IFN-γ assay by the background cavity fluid IFN-γ level and expressed as IU/mL^2.

for those with non-TBE. The median value of IFN-γ in the whole-blood IFN-γ assay was significantly higher for patients with ATBS than for patients with non-TBE, as was the median ADA value. For the whole-blood assay, none of the patients had indeterminate results, and 1 patient with ATBS was unavailable for testing. The ADA assay was not performed for 1 patient with tuberculosis.

Relative discriminative accuracy of the whole-blood, ADA, background IFN-γ, and cavity fluid assays was assessed in the area under the ROC curve (figure 3, table 3). The relative discriminative accuracy of the cavity fluid assay was statistically significantly different from that of the ADA ($P = .037$) and whole-blood assays ($P < .001$). The ROC curve for the cavity fluid assay was bowed further to the upper left, compared with that for the background IFN-γ level, but the areas under the ROC curve for the 2 assays were not statistically significantly different at the 5% level of type I error rate ($P = .74$).

Multivariate logistic regression was used as an aid in assessment of whether the cavity fluid assay added any diagnostic information to that yielded by the background IFN-γ level (table 4). Spearman's coefficient of correlation between the 2 measurements was 85%. The 2 measurements did not display statistical evidence of an interaction ($P = .84$). The Hosmer-Lemeshow test did not indicate a lack of fit ($P = .74$); thus, the predictors provide for a reasonably well calibrated model without data transformation. The cavity fluid assay displayed

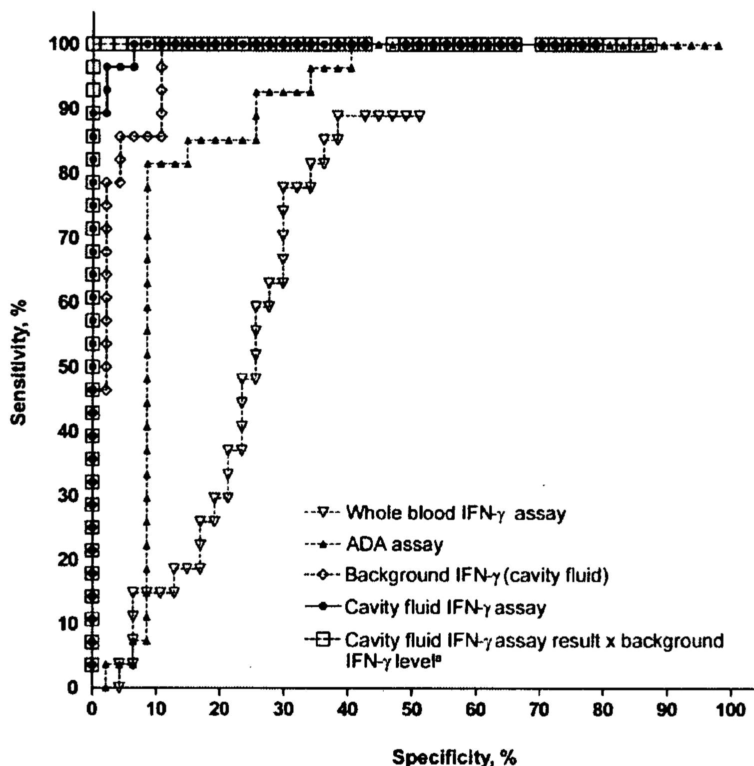

Figure 3. Receiver operating characteristic curves for 5 diagnostic methods used for patients with active tuberculous serositis and nontuberculous effusion. [a]Values calculated by multiplying the result of the cavity fluid IFN-γ assay by the background cavity fluid IFN-γ level and expressed as IU/mL^2.

a statistically significantly nonzero level of association with diagnostic status, even after adjustment for background IFN-γ concentrations, which supports the contention that the cavity fluid assay provides for improved diagnostic accuracy over that provided by the background IFN-γ level alone.

The cutoff value for each test was chosen to maximize specificity without significant loss of sensitivity. Table 5 shows sensitivity, specificity, likelihood ratio, and predictive value by cross classification. To calculate the predictive value, ~3.2% of pretest probability of ATBS in our specialist hospital for tuberculosis was used. As a result, both the likelihood ratio and predictive value of the cavity fluid assay were greater than those of the other assays. Furthermore, the value from when the results of the cavity fluid assay were multiplied by the background IFN-γ level was the most sensitive and specific predictor of ATBS.

DISCUSSION

Area under the ROC curve is the primary index for assessing the discriminative accuracy of a diagnostic method. Using this index, the cavity fluid assay displayed greater ability to discriminate ATBS than did either the ADA or whole-blood assays. The ROC curve index was not able to detect a difference in discriminative accuracy between unstimulated and specific antigen-stimulated IFN-γ response ex vivo. Nevertheless, a diagnostic advantage in assaying specific antigen-stimulated IFN-γ response was evident such that a multivariate logistic regression model provides a better fit to the clinical diagnosis when specific antigen-simulated IFN-γ production is included; the regression coefficient for the cavity fluid IFN-γ assay is statistically significantly different from zero. This better fit indicates improved calibration of the model. It is recognized that

Table 3. Comparison of diagnostic accuracy by area under the receiver operating characteristic (AUROC) curves.

Variable	AUROC curve (95% CI)	SE
Whole-blood IFN-γ assay	0.719 (0.598–0.838)	6.1
ADA assay	0.882 (0.799–0.965)	4.2
Background IFN-γ of cavity fluid	0.975 (0.946–1.004)	1.5
Cavity fluid IFN-γ assay[a]	0.996 (0.989–1.004)	0.4

[a] The value of the cavity fluid IFN-γ assay was defined as the difference between the determined higher IFN-γ value after stimulation with either early secretory antigenic target 6 or culture filtrate protein 10 and the background IFN-γ concentration.

Table 4. Multivariate logistic regression analysis.

Variable	Regression coefficient, log odds per IU/mL	SE	Likelihood ratio[a]	P	OR (95% CI)
Intercept	−3.47	0.79	...	...	...
Background IFN-γ of cavity fluid	0.34	0.23	3.10	.08	1.40 (0.80–2.19)
Cavity fluid IFN-γ assay	0.19	0.08	18.98	<.005	1.21 (1.03–1.42)

NOTE. Two explanatory variables were treated as continuous variables. Standardized ORs (each variable scaled to its interquartile range) were 5.6 for background IFN-γ and 55 for cavity fluid IFN-γ assay. Spearman's coefficient of correlation between the 2 measurements was 85%. The 2 measurements did not display statistical evidence of an interaction ($P = .84$). The Hosmer-Lemeshow test did not indicate a lack of fit ($P = .74$); thus, the predictors provide for a reasonably well calibrated model without data transformation.

[a] Likelihood ratio was determined using χ^2 test statistics and was single-degree-of-freedom.

area under the ROC curve is important but not always optimum in assessment of diagnostic tests, especially for predictive assessment of risk [21].

For the IFN-γ assay using cavity fluid, the IFN-γ concentration was significantly increased after antigen stimulation, compared with the background IFN-γ concentration, for every patient with ATBS (figure 1). It may be speculated that this result was because of further IFN-γ production by antigen-specific T cells in response to stimulation. We have obtained similar results for 30 patients with pleural effusion in whom active tuberculous pleuritis was strongly suspected clinically. Although, in these patients, definitive bacteriological evidence could not be obtained from pleural effusion specimens, antituberculous chemotherapy was effective (data not shown). In contrast, for the majority of 47 patients with effusion of nontuberculous etiology, IFN-γ concentrations did not increase after stimulation with antigens. This was also true for 5 of 47 patients with nontuberculous pleuritis who had relatively high background IFN-γ concentrations (>1 IU/mL).

In 3 of the patients with non-TBE who had a history of prior tuberculosis and in some of those without any clinical history of tuberculosis, IFN-γ concentrations were slightly increased in cavity fluid after stimulation, compared with background IFN-γ concentrations. Similarly, results of the whole-blood assay were positive for all of these patients. It may be speculated that when patients with a history of tuberculosis encounter serositis attributable to causes other than tuberculosis, preexisting antigen-specific memory T cells in peripheral blood also appear in the effusion and produce IFN-γ through stimulation by antigens in vitro. If diagnostic evaluation was made only on the basis of measuring an antigen-specific IFN-γ response or on a count of IFN-γ–producing cells by enzyme-linked immunospot assay [22], there is a possibility that some patients with an effusion of nontuberculous etiology who have incidental latent tuberculosis infection (LTBI) will receive a false-positive diagnosis. In such cases, non-TBE with LTBI can be differentially diagnosed from ATBS on the basis of low background IFN-γ concentration (figure 1). We speculate that high background IFN-γ concentrations in patients with ATBS are a reflection of the ongoing active status of the local type 1 helper T cellular immune response. High IFN-γ concentrations in the supernatant of pleural effusion specimens from patients with tuberculous pleuritis have been reported [3, 7, 23, 24]. However, it has also been demonstrated that the IFN-γ concentration can be high in those patients with an effusion of nontuberculous etiology [7]. In our study, 5 of 47 patients with non-TBE (i.e., *M. avium* pleuritis, cancerous pleuritis, and parapneumonia) had background IFN-γ concentrations that were similar to those found in patients with ATBS (>1 IU/mL). Thus, it appears that specificity of the background IFN-γ level may be limited and that false-positive responses can occur. The cavity fluid assay can assess both background IFN-γ and antigen-specific IFN-γ responses simultaneously, and it is possible to compensate for the fault mutually by assessing the value of both responses together. We demonstrated that the values from when the result of the cavity fluid assay was multiplied by the result of the background IFN-γ possibly could be used to accurately diagnose active tuberculous effusion (figure 2, table 5).

Although both the enzyme-linked immunospot assay using the peripheral blood and whole-blood IFN-γ assay have been reported for the diagnosis of tuberculosis, the results of these assays were also positive for people with LTBI. The whole-blood assay also has been reported to be highly specific for *M. tuberculosis* infection, but it cannot discriminate between active tuberculosis and LTBI. In our study, the group of patients who did not have ATBS likely contained a number of subjects with LTBI. Indeed, 3 patients with non-TBE who had a documented history of prior tuberculosis had positive whole-blood assay results. Similarly, 11 other patients who did not have ATBS had positive whole-blood assay results but had no clear history of tuberculosis or evidence of an old tuberculous lesion on chest radiograph (table 5). Because of the high specificity (>98%) of the whole-blood IFN-γ assay among a young population with no risk for tuberculosis [19], we speculated that these patients were latently infected with tuberculosis, reflecting their age and an era when tuberculosis was prevalent in Japan. An additional

Table 5. Comparison of diagnostic accuracy of adenosine deaminase (ADA), whole-blood, and cavity fluid IFN-γ assays.

Variable	Cutoff value	Result, positive: negative ATBS group	Non-TBE group	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive likelihood ratio	Negative likelihood ratio	PPV[a]	NPV[a]
Whole-blood IFN-γ assay	0.281	21:6	14:33	77.8 (57.7–91.4)	70.2 (55.1–82.7)	2.6	3.2	7.94	99.0
ADA assay	40.700	22:5	4:43	81.5 (61.9–93.7)	91.5 (79.6–97.6)	9.6	4.9	24.5	99.3
Background IFN-γ of cavity fluid	2.456	24:4	2:45	85.7 (67.3–96.0)	95.6 (85.5–99.5)	20.1	6.7	39.7	99.5
Cavity fluid IFN-γ assay[b]	2.352	27:1	1:46	96.4 (81.7–99.9)	97.8 (88.7–99.95)	45.3	27.4	60.3	99.9
Cavity fluid assay result times the background IFN-γ level[c]	2.590	28:0	0:47	100 (87.7–100)	100 (92.5–100)	...	...	100.0	100.0

NOTE. For the whole-blood assay, none of the patients had indeterminate results, and 1 patient with active tuberculous serositis (ATBS) was unavailable. The ADA assay was not performed for 1 patient with ATBS. Non-TBE, nontuberculous effusion; NPV, negative predictive value; PPV, positive predictive value.

[a] Pretest probability of active tuberculous effusion in our department of respiratory medicine was 3.2%

[b] The result of the cavity fluid IFN-γ assay was defined as the difference between the determined higher value of IFN-γ after stimulation with either early secretory antigenic target 6 or culture filtrate protein 10 and the background IFN-γ concentration.

[c] The values from when the result of the cavity fluid IFN-γ assay was multiplied by the background IFN-γ level.

finding was that the whole-blood assay result was negative for 6 of 27 patients with ATBS. In this study, results of the whole-blood assay showed reduced sensitivity (77.8%), compared with the sensitivity found in previous reports (89%) [19]. It has been reported that T cells that are specific for tuberculous antigens are sequestered from the circulation to the pleural cavity in patients with pleural tuberculosis [22]. The migration of antigen-specific T cells from peripheral blood to the active site of disease was thought to be 1 possible cause of lower than previously reported sensitivity of the whole-blood assay.

ADA in the supernatant of pleural effusion is reported as a diagnostic marker for tuberculous pleuritis [25–27]. The sensitivity and specificity of the ADA assay were reported to be 47.1%–100% and 0%–100%, respectively [28]. In our study, ADA concentrations were significantly higher in patients with ATBS than in patients with non-TBE. With regard to both sensitivity and specificity, the ADA assay had better performance than did the whole-blood assay for diagnosis of ATBS (table 5). However, in 4 of 47 patients with non-TBE (attributable to asbestosis, adenocarcinoma, and parapeumonic pleural effusion), ADA concentrations were as high as those in patients with ATBS. Furthermore, in some patients with active pleural and peritoneal tuberculosis, the ADA concentration was as low as that in patients with non-TBE. These false-positive and false-negative cases will be problematic when physicians make decisions regarding the initiation of long-term antituberculous chemotherapy.

In conclusion, we reported a highly sensitive and specific diagnostic method for active tuberculous pleuritis, tuberculous peritonitis, and pericarditis in which IFN-γ responses were measured after stimulation of cavity fluid cells with *M. tuberculosis*–specific antigens. The cavity fluid IFN-γ assay could be a noninvasive method for accurately and promptly diagnosing tuberculous serositis in patients in whom active tuberculosis in the cavity space is clinically suspected but for which no bacteriological evidence can be obtained.

Acknowledgments

We thank Yurika Ito, Ayako Watanabe, and Yasuko Inoue, for their technical assistance with the laboratory assays, and Drs. Kazuhiro Uchiyama, Toshiki Tamura, and Hajime Goto, for their support.

Financial support. The Ministry of Health, Labor and Welfare of Japan.

Potential conflicts of interest. All authors: no conflicts.

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nature Vol 448|30 August 2007|doi:10.1038/nature06096

LETTERS

Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome

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Hyper-immunoglobulin E syndrome (HIES) is a compound primary immunodeficiency characterized by a highly elevated serum IgE, recurrent staphylococcal skin abscesses and cyst-forming pneumonia, with disproportionately milder inflammatory responses, referred to as cold abscesses, and skeletal abnormalities[1]. Although some cases of familial HIES with autosomal dominant or recessive inheritance have been reported, most cases of HIES are sporadic, and their pathogenesis has remained mysterious for a long time. Here we show that dominant-negative mutations in the human signal transducer and activator of transcription 3 (*STAT3*) gene result in the classical multisystem HIES. We found that eight out of fifteen unrelated non-familial HIES patients had heterozygous *STAT3* mutations, but their parents and siblings did not have the mutant *STAT3* alleles, suggesting that these were *de novo* mutations. Five different mutations were found, all of which were located in the STAT3 DNA-binding domain. The patients' peripheral blood cells showed defective responses to cytokines, including interleukin (IL)-6 and IL-10, and the DNA-binding ability of STAT3 in these cells was greatly diminished. All five mutants were non-functional by themselves and showed dominant-negative effects when co-expressed with wild-type *STAT3*. These results highlight the multiple roles played by STAT3 in humans, and underline the critical involvement of multiple cytokine pathways in the pathogenesis of HIES.

Elevated serum IgE is a hallmark of many allergic disorders[2]. Curiously enough, the hyper-IgE state is also observed in some primary immunodeficiency disorders, such as HIES, Wiskott-Aldrich syndrome, Omenn syndrome and Comèl-Netherton syndrome[3]. HIES (OMIM number 243700) was first reported in 1966 as Job's syndrome (OMIM number 147060)[4,5], but its underlying cause is still unknown, unlike the other three syndromes. In most cases of HIES, the clinical manifestations extend over multiple systems in the body, including the immune system, skeletal/dental system and soft tissue[6]. In contrast, the abnormalities in familial autosomal recessive (AR)-HIES patients seem to be confined to the immune system[7]. We previously identified a homozygous mutation of the tyrosine kinase 2 (*TYK2*) gene in a patient who showed AR-HIES and susceptibility to intracellular bacterial infections[8]. TYK2 is a non-receptor tyrosine kinase belonging to the JAK family[9,10]. The patient's cells expressed no detectable TYK2 protein and displayed defects in multiple cytokine signals, including the signalling pathways for IL-6, IL-10, IL-12, IL-23 and type I IFN. The cytokine signals were successfully restored by introducing the intact *TYK2* gene into the patient's cells. These multiple defects probably account for the patient's complex clinical manifestations[8]. The identification of a TYK2 deficiency in this HIES patient indicated to us that, besides TYK2, one or more molecules shared by multiple cytokine signalling pathways might also cause HIES.

To explore this possibility, we first examined the responses to IL-6, IL-10, IL-12 and IFNα of peripheral blood cells from two patients (patient 1 and patient 2) who showed characteristics of multisystem HIES, including skeletal/dental abnormalities, skin abscesses, cyst-forming pneumonia and highly elevated serum IgE (Supplementary Table 1). The patients' B cells secreted IgM normally when stimulated with Epstein–Barr virus (EBV) infection (Fig. 1a). However, additional stimulation with IL-6 induced no significant increase in IgM secretion in the patients' B cells, unlike in the control B cells (Fig. 1a). Moreover, the suppression of lipopolysaccharide-induced production of TNFα by IL-10 deteriorated in the patients' macrophages (Fig. 1b). Thus, both the IL-6 and IL-10 pathways were defective in these HIES patients, as in the TYK2-deficient patient (Fig. 1a and 1b). In contrast, neither IL-12 nor IFNα signalling was impaired in the HIES patients, unlike in the TYK2-deficient patient (Fig. 1c and 1d). The HIES patients' T cells produced IFNγ normally in response to IL-12 (Fig. 1c), and their peripheral blood mononuclear cells (PBMCs) showed normal upregulation of transcripts for two IFN-inducible genes, *NMI* and *MX1* (also known as *MxA*), in response to IFNα (Fig. 1d). These observations indicated a possible abnormality in one or more molecules that was shared by the IL-6 and IL-10 signals but not essential for the IL-12 and IFNα pathways.

An array of cytokine signals is transduced by different combinations of JAK family kinases and STATs[9,10]. When cytokines bind to their receptors, receptor-associated JAKs are activated to phosphorylate STATs, which in turn dimerize and translocate to the nucleus, where they activate target genes. In a survey of possible abnormalities in this signal cascade, we identified heterozygous mutations in the STAT3 DNA-binding domain in both patients: a single amino acid deletion (ΔV463) in patient 1 and a mis-sense mutation (R382W) in patient 2 (Fig. 2). Because STAT3 is shown to be activated in response to a wide variety of cytokines, growth factors, and hormones[11,12], we thought that its mutation could well account for the patients' complex clinical manifestations extending over multiple systems. Further analysis of the STAT3 complementary DNA sequences in thirteen more unrelated patients with non-familial HIES identified heterozygous mutations in six of these patients (patients 3–8; see Supplementary Table 1 for clinical summary), and all of these mutations were located in the DNA-binding domain of STAT3: ΔV463 in patient 3 and patient 8, like in patient 1; R382Q in patient 4; H437Y in patient 5; T389I in patient 6; R382W in patient 7, like in patient 2 (Fig. 2). The five different STAT3 mutations found in the

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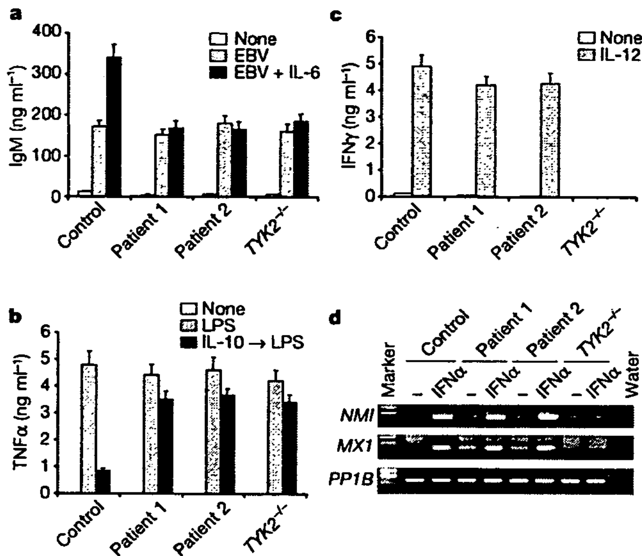

Figure 1 | Impaired responses to IL-6 and IL-10 in HIES patients' cells. **a**, IgM levels in culture medium of PBMCs from a control subject, two HIES patients (patient 1 and patient 2), and the TYK2-deficient patient, cultured for 7 days without stimulation, with EBV alone or with EBV and IL-6. **b**, TNFα levels in culture medium of macrophages from the same subjects, cultured without or with lipopolysaccharide (LPS) stimulation for 48 h, or with IL-10 treatment for 24 h before lipopolysaccharide stimulation. **c**, IFNγ in culture medium of CD4$^+$ T cells from the same subjects, cultured for 24 h without or with IL-12. Error bars show standard deviations (**a–c**). **d**, *NMI*, *MX1* and cyclophilin B (*PP1B*) transcript levels in PBMCs from the same subjects were unstimulated or stimulated with IFNα for 2 h.

eight patients were all confirmed by sequencing their genomic DNA, and no sequence alterations were detected in any other parts of *STAT3*, *TYK2* or *JAK1*. The DNA-binding domain of STAT3 is highly conserved among different species in its amino acid sequence, and the alterations identified in the patients' *STAT3* gene were statistically highly significant in population genetics ($P < 10^{-15}$, Fisher's exact probability test), as judged by the fact that such alterations were not found in 1,000 unrelated healthy individuals analysed, including at least 100 controls from the patients' ethnic group. *STAT3* is located on human chromosome 17q21, but not 4q, which was reported to contain a disease locus for familial AD-HIES[13]. None of the eight HIES patients in the present study had a known family history of HIES, and no mutation was detected in the *STAT3* cDNAs from all the parents and seven siblings of the patients, even though an analysis of multiple polymorphic markers confirmed the biological parent–child relationship (data not shown). Therefore, the mutations are likely to have occurred *de novo* in the HIES patients.

We next evaluated the biological significance of the STAT3 mutations. The STAT3 protein levels were comparable in all the EBV-transformed B-cell lines established from patients 1–6 and a control subject, and the extent of the tyrosine phosphorylation on STAT3 induced by IFNα stimulation was also comparable (Fig. 3a). Furthermore, all the mutant proteins formed a complex with wild-type STAT3 as efficiently as did wild-type STAT3, as judged by the co-immunoprecipitation of wild-type and mutant STAT3 proteins co-expressed in COS7 cells (Fig. 3b). However, nuclear extracts isolated from the patients' cells stimulated with IFNα contained much lower amounts of active STAT3 that could bind to target DNA compared with nuclear extracts from the control cells, whereas the DNA-binding activity of STAT1 was intact in the patients' cells (Fig. 3c). This finding was consistent with a previous report showing that changing codons 461 to 463 in STAT3 from Val-Val-Val to Ala-Ala-Ala resulted in the impairment of STAT3's DNA-binding activity[14]. Thus, the genetic mutations identified in the HIES patients seemed to result in the impairment of the DNA-binding activity of STAT3 and most likely that of heterodimers between mutant and wild-type STAT3 molecules. A similar impairment of DNA-binding activity in STAT1 protein was recently reported in cells isolated from patients carrying a heterozygous mutation in the DNA-binding domain of STAT1 (ref. 15).

When wild-type STAT3 was exogenously expressed—together with a luciferase reporter gene containing STAT3-responsive elements—in human HeLa cells in which the endogenous STAT3 was knocked down, fivefold upregulation of luciferase activity in response to IFNα was detected (Fig. 4a). In contrast, none of the STAT3 mutants conferred any significant increase of luciferase activity on the HeLa cells in response to IFNα, demonstrating a loss of function of the STAT3 mutants. To explore the possibility that the STAT3 mutant proteins function as dominant-negative, wild-type STAT3 or the individual mutants were exogenously expressed in IL-6-responsive HepG2 cells and IL-10-responsive MC/9 cells (Fig.

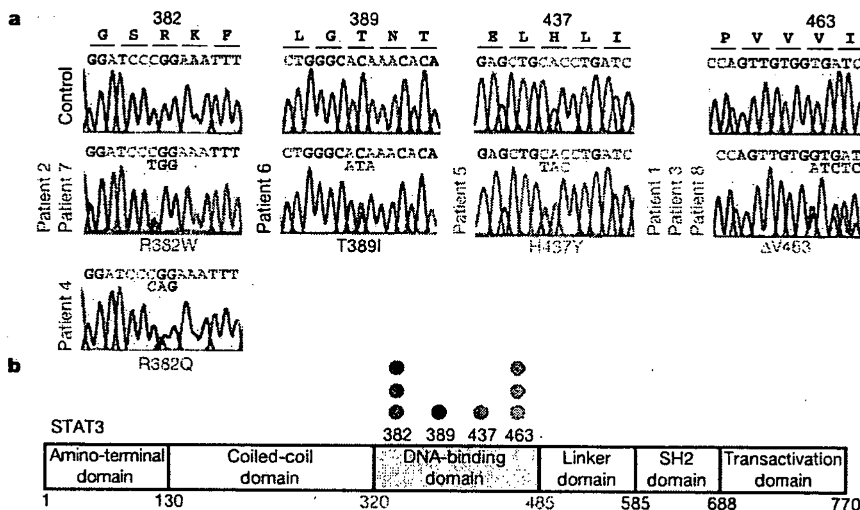

Figure 2 | Heterozygous mutations in the DNA-binding domain of STAT3 from eight HIES patients. **a**, Electropherograms showing partial *STAT3* cDNA sequences from a control subject and the eight HIES patients. **b**, The structure of STAT3 is shown schematically, and the positions of the mutations identified in the eight patients are indicated.

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4b and 4c). In HepG2 cells transfected with empty vector together with the reporter luciferase gene, the luciferase activity was upregulated as much as 3.5 times its basal level in response to IL-6 stimulation. The IL-6-induced upregulation of luciferase activity was augmented in HepG2 cells transfected with wild-type *STAT3* by up to 5.5 times, whereas it was severely impaired in cells transfected with any of the mutant *STAT3*s, showing at most a 2-fold increase (Fig. 4b). Moreover, the IL-10-induced downregulation of surface KIT (C-Kit) expression was severely impaired in MC/9 cells transfected with any of the mutant STAT3s, unlike those with empty vector or wild-type *STAT3* (Fig. 4c). Thus, all the STAT3 mutants identified in the HIES patients displayed dominant-negative effects when co-expressed with wild-type STAT3.

The DNA-binding activity of STAT3 in the IFNα-stimulated patients' cells was not totally abrogated, although it was only approximately one-fourth that of control cells (Fig. 3b). This residual STAT3 activity might have rescued the patients from early embryonic death, which is observed in STAT3-deficient mice[16]. In contrast, its diminished activity might have an impact on the development and functions of multiple organ systems, leading to compound clinical manifestations of HIES, given STAT3's role in the signalling pathways of a variety of soluble factors, including the IL-6-family cytokines (IL-6, IL-11, IL-27, IL-31, LIF, OSM, CNTF and cardiotrophin-1), the IFN-family cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IFNα/β and IFNγ), the IL-2-family cytokines (IL-2, IL-7, IL-9, IL-15 and IL-21), IL-5, IL-23, CSF3/G-CSF, EGF, CSF1 and leptin[12,17,18].

It has been shown that STAT3 has important roles in the differentiation of both osteoblasts and osteoclasts *in vitro*[19], and mice deficient for STAT3 in osteoblasts show an osteoporotic phenotype[20]. When osteoclasts were generated from peripheral blood monocytes in culture with CSF1/M-CSF and TNFSF11/RANKL, those from the HIES patients with the STAT3 mutations showed higher bone-resorption activity compared to those from control subjects (Supplementary Fig. 1). This may reflect the skeletal/dental abnormalities

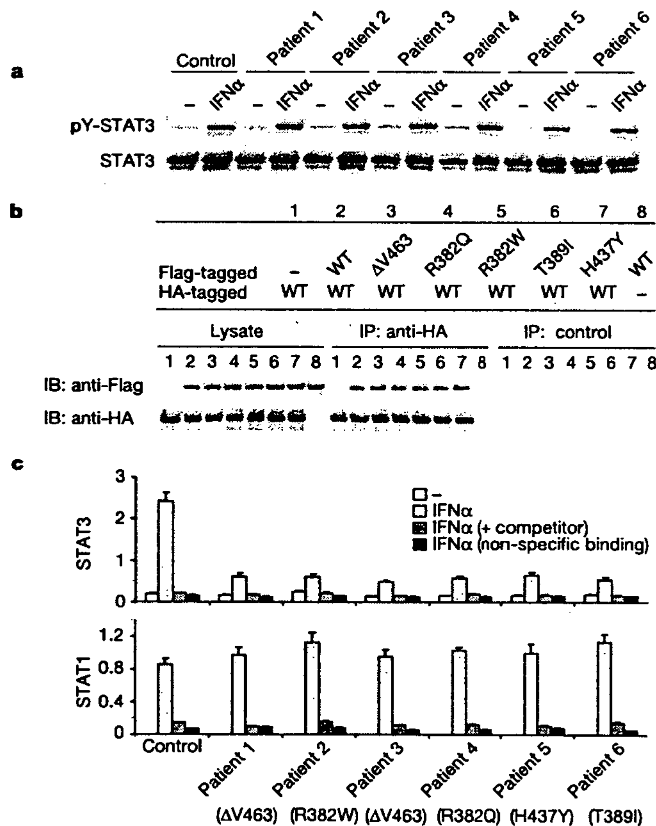

Figure 3 | Diminished DNA-binding activity of STAT3 in the HIES patients' cells. **a**, Total and tyrosine-phosphorylated (pY-) STAT3 proteins detected by immunoblotting. EBV-transformed B-cell lines (EBV-LCL) established from a control subject and the HIES patients were unstimulated or stimulated with IFNα for 15 min. **b**, Association of Flag- or HA-tagged wild-type (WT) and mutant STAT3s co-expressed in COS7 fibroblast cells was examined by immunoprecipitation (IP) with anti-HA or control antibody followed by immunoblotting (IB) with anti-Flag or HA antibody. **c**, DNA-binding activity of STAT3 and STAT1 in nuclear extracts prepared from EBV-LCLs from a healthy control and the HIES patients that were unstimulated or stimulated with IFNα for 15 min. Dark grey bars indicate the DNA-binding activity in the presence of competitor oligonucleotides, and black bars indicate non-specific binding to irrelevant oligonucleotides. Error bars are standard deviations.

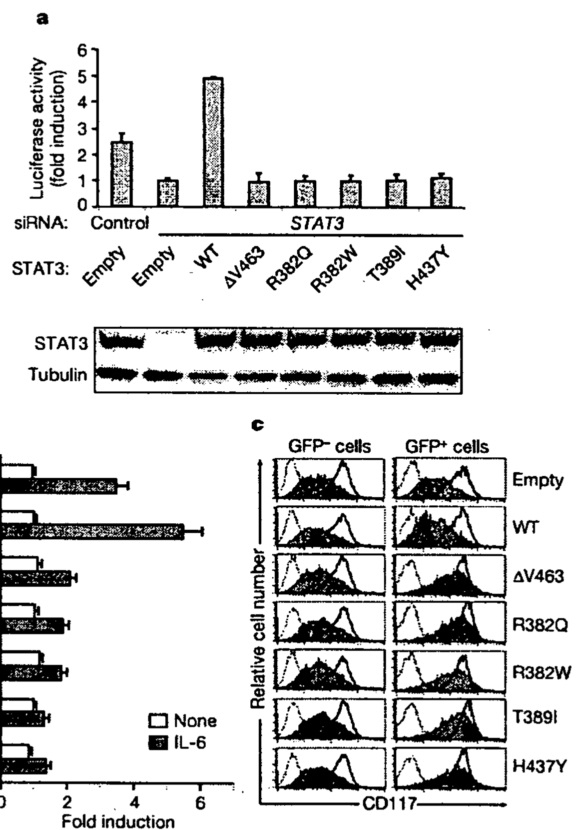

Figure 4 | Loss-of-function and dominant-negative effect of the STAT3 mutants in cytokine signals. **a**, Wild-type or individual mutants of STAT3 were exogenously expressed together with a luciferase reporter gene containing STAT3-responsive elements in human HeLa cells in which endogenous *STAT3* was knocked-down by transfecting with two sets of siRNA oligonucleotides. The HeLa cells were then left unstimulated or stimulated with 10 ng ml^{-1} of IFNα for 5 h. The relative luciferase activity in the cell lysates of IFNα-stimulated versus unstimulated cells is shown (error bars show standard deviations). Expression of endogenous and exogenous STAT3 proteins detected by immunoblotting is shown in the lower panel. **b**, The relative luciferase activity in the cell lysates of unstimulated or IL-6-stimulated HepG2 cells that were transfected with the luciferase reporter construct plus an empty vector or a vector containing the wild-type (WT) or one of each mutant *STAT3* sequence. Error bars show standard deviations. **c**, The CD117 expression level on untreated (thick line histograms) or IL-10-treated (shaded histograms) MC/9 cells that were infected with retroviral vectors containing WT or one of the mutant *STAT3* sequences. Data are shown for GFP^{+} infected and GFP^{-} uninfected cells, and thin dotted histograms indicate staining with a control antibody.

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