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Chapter 13: Immunological Diagnosis

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13.1. Historical Overview

The gold standard for tuberculosis (TB) diagnosis is the demonstration of mycobacteria in various body fluids. This is often not possible, due to the paucibacillary nature of the illness in some cases, for example in children. On the other hand, microscopic identification and culture of mycobacteria in sputum are the most common methods for diagnosis of pulmonary disease, but the detection of extrapulmonary TB is often more difficult. In the search for rapid and cost-effective diagnostic methods for TB, immunodiagnosis is considered an attractive option. Basically, it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease. The tuberculin skin test (TST) (Huebner 1993) and, more recently, the antigen-specific *ex vivo* induction of interferon-gamma (IFN- γ) production have been used to detect infection with *Mycobacterium tuberculosis* (Pai 2004). At the same time, a wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have also been evaluated to detect active disease (Gennaro 2000, Chan 2000). Serology has additional advantages in situations when:

- the patient is unable to produce adequate sputum
- sputum smear results are negative
- TB is extrapulmonary

13.1.1. Serological diagnosis

Historically speaking, serology for the diagnosis of TB has been explored since 1898, when crude cell preparations containing carbohydrates, lipids, and proteins from *M. tuberculosis* or *M. bovis* BCG were used as antigen preparations showing high sensitivity but low specificity (Arloing 1898). Modern developments in the purification of antigens, generation of monoclonal antibodies and chromatographic techniques, have led to a considerable improvement in specificity. During the last three decades, a large number of purified (native and recombinant) antigens have been assessed, showing substantial progress in the serodiagnosis of TB (Jackett 1988).

13.1.2. The century-old skin test for detection of latent tuberculosis

In 1882, about eight years after the discovery of the tubercle bacillus, Robert Koch announced a cure for TB. He obtained a heat-inactivated filtrate from cultures of *M. tuberculosis*, and found that this material would protect guinea pigs from experimental TB. This product, known as “Koch’s Old Tuberculin”, was then administered to patients with TB, and Koch claimed that this treatment resulted in the cure of the disease (Kaufmann 2000, Gradmann 2001, Gradmann 2006). However, TB patients who received tuberculin had generalized systemic reactions, including fever, muscle aches, and abdominal discomfort with nausea and vomiting, in contrast to people without TB, who did not develop this violent reaction. These observations were the basis for the proposal of the use of tuberculin as a diagnostic test, despite its failure as a therapeutic substance. The intradermal injection of tuberculin was described by Mantoux, and his method became widespread because of the reproducibility of the results. After local application of the product injected intradermally, a hallmark response is elicited within 24 to 72 hours, which includes induration, swelling and monocytic infiltration into the site of the injection (Figure 13-1). The skin reaction, classified as delayed type hypersensitivity (DTH), has been used since then to test if prior exposure to an antigen has occurred. Koch's tuberculin was an impure extract of boiled cultured tubercle bacilli. In 1934, Siebert made a simple protein precipitate of the old tuberculin and named it purified protein derivative (PPD) (Figure 13-2).



Figure 13-1. Tuberculin skin test.



Figure 13-2. Florence Seibert.

13.2. Current methods of tuberculosis diagnosis

An overview of the current general methods of TB diagnosis is given in Table 13-1.

Table 13-1: Current methods of TB diagnosis (© LIONEX, Braunschweig)

Method	Advantages	Disadvantages
Clinical signs	Rapid diagnosis	Not specific, not conclusive Not always present
X-Ray	Readily available	Not specific or conclusive
Microscopy (smear for acid-fast bacilli)	Low cost Rapid diagnosis	Low sensitivity (up to 2/3 of pulmonary TB cases are negative) Difficult sample collection
Culture	Specific	Time consuming (up to 4-8 weeks) Not always possible
PCR	Relatively quick Very specific	Relatively expensive High level of training required Expensive instrumentation Can detect latent disease

Method	Advantages	Disadvantages
BACTEC	Specific	Slow, 2-3 weeks Expensive Not possible in all cases
Tuberculosis ELISA test kits	Quick (procedure time: 110 min) Reproducible Minimal training	Some equipment required
Rapid tests	Very quick (procedure time: only 15 min) Minimal training No special equipment required	Lower sensitivity compared to ELISA test kits No quantitative results

13.3. Basis of immunological diagnosis

There is strong evidence both *in vivo* and *in vitro* of cellular immune reaction in TB patients and in those infected with *M. tuberculosis*. *In vivo*, this reaction can be measured by DTH response to PPD; and *in vitro*, by the proliferation of lymphocytes to different compounds of the bacteria. (Lalvani 1998, Pai 2004). In the last decade, extensive studies have shown that immunodominant antigens, such as the 6-kDa early secretory antigenic target (ESAT-6) and its homologues, are highly suitable for detecting infection. There is no cross-reaction with the BCG vaccine, since these antigens are absent in the BCG vaccine strains.

On the other hand, there have been several studies showing humoral response to antigens in patients suffering from active disease (Bothamley 1994, Wilkinson 1997). But the general view in the scientific community has been that the specificity and the magnitude of the humoral immune response are inadequate from the point of view of TB diagnosis. The majority of studies have concentrated on smear-positive TB with very little emphasis on smear-negative TB disease, which may account for 30-60 % of the cases depending on the prevalence of TB (Jackett 1988). Also, smear-negative TB disease is the future target for serodiagnosis, because smear-positive TB cases are easily diagnosed by microscopy.

13.3.1. Humoral immune response

Early studies utilizing crude antigen preparations of *M. tuberculosis* showed sero-reactive antibodies in TB patients. However, cross-reactions occurred in healthy individuals, elicited by commensal bacteria, environmental mycobacteria and BCG vaccination (Bardana 1973, Laal 1997). During the last two decades, the antibody

response to purified antigen preparations of 38 kDa (PhoS), 30/31 kDa (antigen 85, 19 kDa lipoprotein, 14 kDa, 16 kDa (ACR) and lipoarabinomannan (LAM) was also tested. The 38 kDa antigen has shown the highest sensitivity and specificity (Wilkinson 1997, Espitia 1989). It is interesting to note that anti-38 kDa antibodies seem to be restricted to advanced TB (Jackett 1988, Davidow 2005), which is the main cause of TB transmission. There are also reports on antigens (16 kDa and 88 kDa) to which antibodies are generated in the early stages of the disease (Laal 1997).

There seems to be some association of TB and TB-specific antibody levels with human leukocyte antigens (HLA) (Bothamley 1989), which may be responsible for a heterogeneous humoral immune response to TB antigens in human populations. While augmenting the humoral immune response, some genes suppress the spontaneous and antigen-induced lymphocyte response in DR2-positive patients with active disease (Selvaraj 1998).

Weldingh *et al.* (Weldingh 2005) performed a detailed study on 35 antigens of *M. tuberculosis* proteins that are absent in BCG. The authors reported the identification of additional antigens useful for improving the sensitivity of serodiagnosis in African populations. But further studies are needed to confirm these results.

A number of studies with purified native or recombinant antigens have agreed that in order to produce a useful serological tool for TB diagnosis, several antigens must be combined as a cocktail mixture. Several decades of published literature on a series of expression systems and applications also show that fusion proteins can be produced incorporating several antigens using the standard recombinant DNA technology. There is nothing novel in this kind of approach.

One must note that all published work on individual antigens or mixtures of antigens has been produced by individual research groups and there has been no confirmation of these results by other groups, except those concerning antigens such as 38 kDa and 16 kDa. Even in these cases, wrong interpretations have been stated, most probably due to the insufficient information available on the size, solubility and chemical confirmation of a given antigen. In our view, this aspect of a protein antigen candidate for studying serological response is of utmost importance. Another serious flaw in published studies is the almost complete omission of the influence of human populations (TB and healthy) from different geographic regions on the immune response to a particular antigen.

13.3.2. Cellular immune response and delayed type hypersensitivity

In 1964, Mackness established unambiguously that immunity to *M. tuberculosis* and to certain other facultative intracellular bacterial pathogens was cell-mediated (Mackness 1964). The cellular immune response comprises the activation of T helper lymphocytes (CD4+) and cytolytic T lymphocytes (CTL) or killer T lymphocytes (usually CD8+). These two types of effector T lymphocytes play a critical role in eliminating or controlling chronic microbial infections. Activated CD4+ T cells can differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells that secrete specific subsets of cytokines. In general, Th1 cells secrete the cytokines IFN- γ and tumor necrosis factor alpha (TNF- α), whereas Th2 lymphocytes secrete the cytokines IL-4, IL-5, IL-10, and IL-13. The Th1 immune response is associated with a strong, cell-mediated CTL response whereas the Th2 response is characterized by a humoral or antibody-mediated immune response (Esser 2003). In addition to providing cytokines for the development and maintenance of a strong CTL response, the IFN- γ and TNF- α secreted by the Th1 cells can have direct lytic effects on intracellular parasites such as *M. tuberculosis*. The induction of a Th1 immune response is dependent on another cytokine, IL-12, which is produced by macrophages and dendritic cells (Flynn 2001).

DTH was also previously found to be a reaction mediated by the cellular arm of the immune system (Landsteiner 1942). For a long time, the role of DTH in cell mediated immunity has been the subject of contentious debate. This reaction has been shown to be absolutely dependent on the presence of memory T cells. Both the CD4+ and the CD8+ fractions of T cells have been shown to modulate a response. The contemporary debate regarding the reaction focuses on the role of the Th1 and Th2 cells. It has been postulated that the Th1 cell is the "inducer" of the DTH response, since it secretes IFN- γ , a potent stimulator of macrophages, while the Th2 cell is either not involved or else acts as a downregulator of the cell mediated immune response. Despite the early experimental success of this theory, further studies have shown that Th2 cells may be involved in certain types of proinflammatory cell mediated immunity (Black 1999). A positive DTH reaction to PPD reflects the triggering of a repertoire of T-cell clones different from those involved in a protective immune response. Indeed, Pais *et al.* have demonstrated that T cells involved in protection to a challenge with live TB bacilli recognized predominantly low-mass culture filtrate antigens below 15,000 MW, while cells recruited in the DTH to PPD were directed to molecular mass fractions between 15,000 and 31,000 MW (Pais 1998).

13.4. Serological assays

13.4.1. Enzyme Linked Immunosorbent Assay (ELISA)

In TB patients, the serological response to mycobacterial antigens has been primarily evaluated using standard ELISA with *in house* methodologies and protocols which certainly differ from laboratory to laboratory. A general outline of the ELISA procedure is shown in figure 13-3.

Few commercial tests based on the detection of specific antigens, such as the 38 kDa protein, have been developed and have been in use, primarily in developing countries (Wilkinson 1997, Lionex available at <http://www.lionex.de/content/inhalt06.htm>). There is still a need to improve the sensitivity or specificity of commercial serological tests.

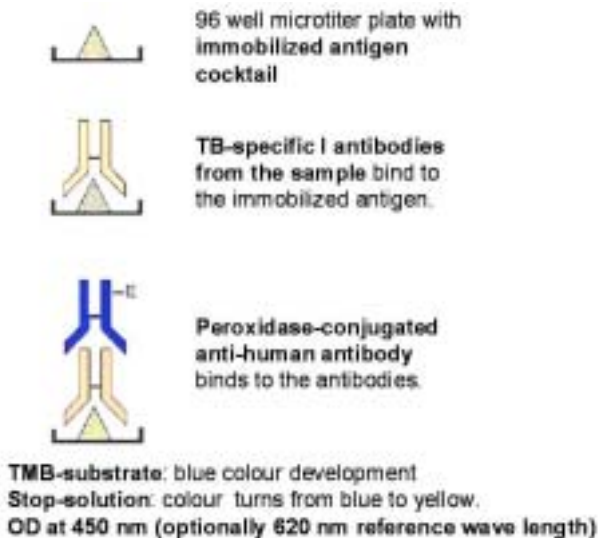


Figure 13-3: General outline of the ELISA procedure.

13.4.2 Rapid tests

Immunochromatographic assays, also called lateral-flow tests or simply strip tests are a logical extension of the technology used in latex agglutination tests. The benefits of immunochromatographic tests include:

- User-friendly format
- Very short time to test result
- Long-term stability over a wide range of climates
- Relatively inexpensive to make

These features make strip tests ideal for applications such as home testing, rapid point-of-care testing, and testing in the field. In addition, they provide reliable testing that might not otherwise be available to low-resource countries.

An example of test procedure (Figure 13-4)

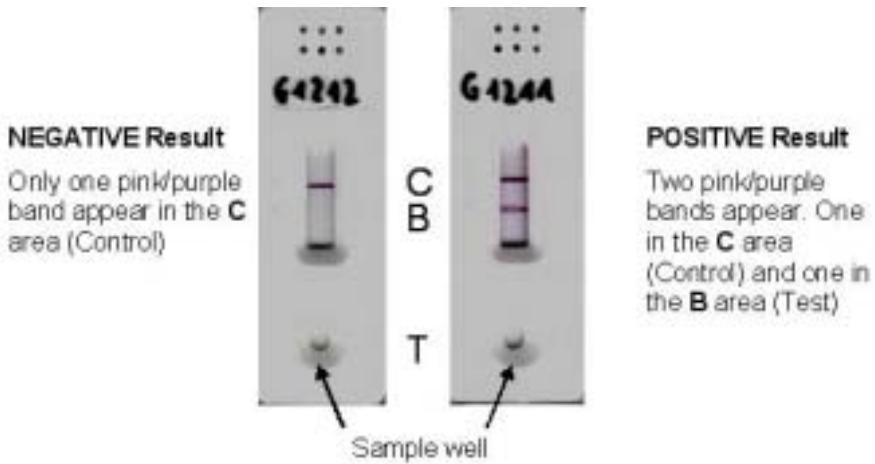


Figure 13-4: Rapid test procedure.

- Remove as many test cards from the pouches as needed. Lay on a clean flat surface.
- Add 40 μl of serum or plasma sample to the T (Test) area of the test card using a measuring pipette or add 1 drop of sample to the T (Test) area by using the provided pipette (included in each test pack). Sample volume for whole blood or EDTA blood: 20 μL .
- Follow sample addition with 2 drops of the diluent provided in the dropper bottle by holding the bottle vertically over the T (Test) Area.
- Results are then read in as little as 20 minutes.

13.5. T cell based assays

One third of the total world's population - two billion people - is believed to be latently infected with *M. tuberculosis*. Latently infected individuals have a 10 % lifetime risk of developing the disease, and this huge global reservoir of infection and disease serves as a continuous source of transmission. Since *M. tuberculosis* is sometimes difficult to culture from patients with active TB, and impossible to culture from latently infected healthy people, it is therefore vital to have efficient tools for diagnosis of active TB and screening for latent *M. tuberculosis* infection. The only widely used test is the century-old TST, based on the intradermal injection of PPD, a crude mixture of *M. tuberculosis* proteins widely shared among *M. tuberculosis*, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), and most environmental mycobacteria (Andersen 2000). Hence, false-positive results are common in people exposed to environmental mycobacterial and/or previously vaccinated with BCG. Thus, there has been an intensive search for specific *M. tuberculosis* antigens that are not cross-reactive with BCG (Mori 2004, Agaard 2004).

By screening eluted fractions of antigens from *M. tuberculosis* and *M. bovis* culture filtrates for recognition by T cells from infected humans and cattle, respectively, Andersen and co-workers identified several low-molecular mass antigens that are major targets of cellular mediated immune responses (Sorensen 1995). Subtractive DNA hybridization of pathogenic *M. bovis* and BCG (Mahairas 1996) and comparative genome-wide DNA microarray analysis of *M. tuberculosis* H37Rv and BCG (Behr 1999) identified several regions of difference, designated RD1 to RD16, between *M. tuberculosis* and *M. bovis* (see Chapters 2 and 8). All represent segments that have been deleted from the *M. bovis* genome. RD1 was lost early during the process of *M. bovis* BCG attenuation and is therefore missing in all the

daughter strains known today (Mahairas 1996). This region has been the subject of detailed studies and a number of antigens have recently been characterized as candidate antigens for diagnostic and vaccine development (van Pinxteren 2000, Andersen 2000, Brusasca 2001). Antigens, such as early secreted antigen target (ESAT-6) and culture filtrate protein (CFP-10), are located in this region and have already shown great potential for TB diagnosis (Ulrichs 1998, Ravn 1999, Arend 2000, Brock 2001).

13.5.1. Tuberculin skin test

TST has been used to identify patients actively infected with TB, to measure the prevalence of infection in a community, and to select susceptible or high-risk patients for BCG vaccination. The test has been in existence for more than 100 years and has remained more or less unchanged for the last 60 years (Huebner 1993, Curley 2003).

TST works by intradermally injecting 0.1 mL of 5 TU PPD on the forearm. On examination, after 48-72 hours, a positive reaction is indicated by erythema and induration of > 10 mm in size. Erythema (redness) alone is not taken as a positive reaction. All persons with prior infection with tubercle bacilli will mount an immune response to bacilli proteins (Curley 2003).

As the active ingredient used in the skin test contains a whole series of proteins that are shared with the BCG vaccine and other mycobacteria common in the environment, the skin test is often falsely positive. It is currently estimated that almost one third of people positive to TST do not actually have TB infection. The sensitivity of the skin test is estimated to be around just 70 % in known active TB cases; so the test misses up to 30 % of people who are infected. This sensitivity decreases to as low as 30 % in immunocompromised people.

TST is difficult to administer correctly, as small variations in the way it is performed vary the amount of PPD delivered into the skin and thus, the resulting size of the reaction. Furthermore, the measurement of the reaction is highly subjective; the variations in diagnosis based on different clinicians reading the same bump in different ways is well documented.

A common problem in those people who are regularly screened for TB infection using the skin test (e.g. healthcare workers) is that they start to become immunized to PPD by its repeated administrations. This is called 'boosting' and results in a false positive reaction to the skin test (a detailed description of the PPD test is

available on the internet at the Medline Plus Medical Encyclopedia <http://www.nlm.nih.gov/medlineplus/ency/article/003839.htm>).

13.5.2. Interferon-gamma determination

One of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seems to be the assays based on IFN- γ determination. The assays stem from the principle that T cells of sensitized individuals produce IFN- γ when they re-encounter the antigens of *M. tuberculosis* (Tufariello 2003). Recent evaluations showed that IFN- γ assays that use *M. tuberculosis* RD1 antigens, such as ESAT6 and CFP10, may have advantages over tuberculin skin testing (Arend 2000, Brock 2001, Lalvani 2001).

IFN- γ assays that are now commercially available are: the enzyme-linked immunospot (ELISPOT) T SPOT-TB assay (Oxford Immunotec, Oxford, United Kingdom), the original QuantiFERON-TB, and its enhanced version QuantiFERON-TB Gold assay (Cellestis International, Carnegie, Australia).

13.5.2.1. Enzyme-linked immunospot for interferon-gamma

The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells specific for *M. tuberculosis* antigens. IFN- γ released *ex vivo* from these cells can be detected by the extremely sensitive ELISPOT (Lalvani 1998). Each such T cell gives rise to a dark spot and the readout is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen *in vivo* and can rapidly release IFN- γ when re-exposed to the antigen (Kaech 2002). In contrast, the long-life memory T cells, which persist long after clearance of the pathogen, are relatively quiescent and less likely to release IFN- γ during the short period of exposure to antigen in the *ex vivo* ELISPOT assay (Lalvani 1998).

Lalvani *et al.* developed the first generation of new ELISPOT tests for latent TB by using the ESAT-6 peptide to stimulate single blood samples. This test detects as few as one in 60,000 IFN- γ producing cells. In a preliminary trial, this test was positive in 96 % of 47 TB patients and in 85 % of 26 persons presumed to have latent TB. The ELISPOT test was negative in 26 BCG-vaccinated control subjects, and this specificity implies a major advantage over TST (Lalvani 2001). The assay has been evaluated by different groups (Lalvani 2001a) and the results have shown that ELISPOT offers a more accurate approach than TST for the identification of individuals who have latent TB infection. These tests could improve TB control by more precise targeting of the preventive treatment.

A commercial ELISPOT test, T SPOT-TB® (Oxford Immunotec, Oxford, United Kingdom) is now available. Related information can be found on the internet at http://www.finddiagnostics.org/news/presentations/lbti_mar_2006/lalvani.pdf.

13.5.2.2. Quantiferon-TB test

QuantiFERON-TB® and Bovigam® are two registered products which measure the release of interferon-gamma in whole blood from human subjects and cattle infected with *M. tuberculosis* and *M. bovis* respectively, in response to stimulation by PPD. The IFN- γ secreted by T-cells into the plasma is measured by ELISA to indicate the likelihood of TB infection. Different studies demonstrated that the QuantiFERON-TB test was comparable to TST in its ability to detect latent TB infection. These studies also showed that the QuantiFERON-TB test was less affected by BCG vaccination, discriminated responses due to non-tuberculous mycobacteria, and also avoided the variability and subjectivity associated with administering and reading the skin test (Pottumarthy 1999).

QuantiFERON-TB® was approved by the Food and Drug Administration (FDA) of the United States (US) in 2001. In 2003, the US Centers for Disease Control and Prevention released guidelines for using the QuantiFERON®-TB Test in the diagnosis of latent *M. tuberculosis* infection, which can be found on the internet at <http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5202a2.htm>. The manufacturer's Quick Reference Guide to the procedure can be found on the internet at <http://www.cellestis.com/IRM/Company/ShowPage.aspx?CPID=1161>.

More recently, an evaluation of the whole blood IFN- γ test for TB diagnosis, based on the specific antigens ESAT-6 and CFP-10, showed that the recombinant antigens could increase the specificity of the whole blood test and enhance the discriminative power of the test between TB infection, atypical mycobacterial reactivity, and reactivity due to BCG vaccination (van Pinxteren 2000).

New, potential T-cell antigens and mixtures of antigens are being evaluated in both human and bovine TB, thus, IFN- γ assays based on these antigens appear to be promising (Aagaard 2004, Aagaard 2006, Leyten 2006). A new product, QuantiFERON®-TB Gold, which includes the TB-specific antigens ESAT-6 and CFP-10 that are only present in *M. tuberculosis* and are absent from all strains of *M. bovis* (BCG) and most environmental mycobacteria, has been introduced. QuantiFERON®-TB and QuantiFERON-TB Gold assays are manufactured by Cellestis International (Carnegie, Australia, <http://www.cellestis.com/>). The antigens used in the Gold version are provided by the Statens Serum Institute in Denmark (<http://www.ssi.dk/sw162.asp>).

13.6. Conclusions and Perspectives

Despite a large number of studies published over the past several years, serology has found little place in the routine diagnosis of TB, even though it is rapid and does not require specimens from the site of disease. Sensitivity and specificity depend on the antigen used, the gold standard used for the diagnosis of TB, and the type of TB disease. Though most of these tests have high specificity, their sensitivity is poor. In addition, these tests may be influenced by factors such as age, prior BCG vaccination and exposure to non-tuberculous mycobacteria strains.

In contrast, while the initial results of IFN- γ determination for the detection of latent infected individuals appear promising, it remains to be seen whether this will translate into practically useful results in the field (Sharma 2006). Indeed, IFN- γ assays are expensive tests and their higher cost appears to limit their wider applicability, especially in resource-limited settings and developing countries, where TB is highly rampant. The ELISPOT test is not yet suitable for widespread use, because it is costly and requires isolation of mononuclear cells, a procedure that is not performed in clinical laboratories.

Because most mycobacterial epitopes are recognized in the context of specific HLA antigens, the IFN- γ based assays should be evaluated at multiple geographic locations, among patients of different ethnicities. Although BCG vaccination does not yield false-positive results in IFN- γ assays using selected antigens, the specificity of the test should be studied in persons exposed to environmental mycobacteria, such as members of the *M. avium* complex. Studies with larger numbers of TB patients are needed to address this issue. The diagnosis of latent TB represents a major advance in the quest for better tests. The explosion of microbial genomics, proteomics, and transcriptomics will yield more *M. tuberculosis* specific genes and antigens; and IFN- γ assays, using peptides from multiple antigens, should be more sensitive than the ones using only ESAT-6 or/and CFP10.

Until we find a reliable diagnostic test for detecting active disease, “TB or not TB?” shall remain a question.

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