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BACTERIA AS PROTEIN CARRIERS IN AGGLUTINATION ASSAYS

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We report here the application of bacteria Brucella abortus as a particulare solid support that can be used as a protein carrier. Carbohydrate unitis of the bacterial surface were oxidised with NaIO4 and human IgG molecules were covalently boud to the bacterial surface. Bacteria coated with human IgG were allowed to react with sheep anti human IgG serum in an agglutination assay. The results of the agglutination test confirmed that satisfactory activition of the bacterial and immobilization of human IgG can be achieved.

Key words: Bacteria, solid phase, Human IgG, Agglutination

INTRODUCTION

Particulate solid phases are widely used for the quantitative determination of biological materials; i. e. polysaccharides, latex particles (Cambiaso et al., 1990; Kondo et al., 1990; Bernard et al., 1991) liposomes (Frost et al., 1990) and different cells (Davis et al., 1991). As a possible solid phase, bacteria have been under investigation mainly as a support carrying immunoglobulins. Teodorescu et al.(1979) and Bratescu et al. (1980) made an identification scheme for lymphocyte subpopulations using antibody coated bacteria after prior tretment of the bacteria with glutaraldehyde. Uchiyama et al. (1985) immobilized IgG-F(ab')2 to paraformaldehyde cross-linked bacterial cell surface in a similar manner.

In our work we investigated covalent linkage of immunoglobulins to the bacterial surface via periodate oxidation of polysaccharide units. Human IgG (hIgG) as an antigen and sheep anti human IgG antiserum (anti-hIgG) were used in a model system to illustrate the application of the obtained protein carrier in the agglutination immunoassay.

MATERIALS AND METHODS

Preparation of bacterial suspension. Brucela abortus 99 WS grown in the laboratories of the Military Medical Academy were used for this study. The bacterial suspension was prepared as described by Bratescu et al. (1980). In order to avoid the risk of bacterial infection, the organisms were killed chemically

with phenol-saline solution (Lako, 1992). Approximately 3.05 x 10^{10} bacteria/ml were used for the coating procedure. The amount of bacteria was determined spectrophotometrically as follows: O. D. value (550 nm) of 0.23 corresponds to 8.0 x 10^8 bacteria/ml. (Teodorescu et al., 1977).

Coupling of bacteria and hIgG. The bacterial suspension (1 ml) was oxidised with different concentrations of NaI04 in water (1 ml: 0.1M, 0.2M, 0.4M, 0.6M and 0.8M) for a period of 2 h, 3 h, 6 h, and 18 h, on a rotator at room temperature (20°C). After oxidation, the suspension was centrifuged at 6000 x g for 10 min and the pellet was washed three times with 0.05M phosphate buffer-0.9% Na Cl, pH 6.9 (PBS). Bacteria were resuspended in 1 ml PBS and 1ml of the hIgG solution was added. hIgG was isolated from human serum by a standard procedure (INEP-Zemun). Different concentrations of hIgG were examined: 0.1 mg/ml, 1.0 mg/ml, 10.0 mg/ml, 20.0 mg/ml, 30.0 mg/ml and 40.0 mg/ml. hIgG coated bacteria were incubated at 4°C and 20°C for 2 h, 3 h, 6 h and 18 h. Coated bacteria were sediment by centrifuging at 6000 x g for 10 min and washed three times with 0.05M PBS. A working suspension was made by resuspending the hIgG coated bacteria in 5 ml of 0.05M PBS-0.01% NaN_3 and, after discarding aggregates formed during the immobilization, by centrifuging the suspension at 3000 x g for 5 min. Bacteria were stored at 4° C until use.

Aggregate analysis — modifications of the coupling procedure. Bacteria were oxidised and coated with hIgG under optimal conditions: 0.6M NaIO4 for 6 h and 30.0 mg/ml hIgG at 20° C for 18 h. Aggregates formed during the overall reaction were separated at 30 x g and 300 x g for 5 min and analysed under the microscope (A).

Procedure A was modified in two ways. First, following the oxidation, the bacterial suspension was centrifuged at 30 x g and 300 x g, for 5 min each, and the remaining suspension was submittet to further coating with hIgG (B).

The second modification occurred at the washing level: instead of washing the hIgG coated bacteria with 0.05M PBS, we applied (1) 0.05M PBS-0.5 BSA (C) and (ii) 0.05M PBS-0.5% TWEEN 20 (D).

Bacterial suspensions that resulted from the modified coupling procedures were analysed in the same manner as the standard bacterial suspension.

Agglutination assay. The assay was carried out in 96 U shaped well plates (TITEREK, Flow Lab., USA) using 25 μl of the suspension of the hIgG coated bacteria and 25 μl of the serially diluted sheep anti-hIgG antiserum (titre of the antiserum was 3.26 g/l; dilutions were prepared in 0.05 M PBS). The reagents were mixed by shaking and the plate was left at 4° C overnight to allow the reaction to occur. The results were expresed as the most diluted antibody concentration as which a visible agglutination occured.

Measurment of the coating efficiency. hIgG (5μg/ml) was labelled with ^{125}I (1mCi, CIS, Gif sur Yvette, France) by the chloramine T method (Hudson and Hay, 1989). ^{125}I-hIgG was used as a tracer to measure the amount of hIgG bound to the bacteria. The intial concentration of hIgG was 30.0 mg/ml. Radioactivity of the bacterial suspension was detected on a γ-scintillaction counter (Gammachem 4800).

hIgG coated bacteria in the reacton with standard anti Brucella abortus antiserum Unoxidised and hIgG coated *Brucella abortus* (initial concentration of the hIgG for the coating was 30.0 mg/ml) were analysed with the standard anti *Brucella abortus* antiserum (Lako, 1992). The bacterial suspension (25 μl) and the serially diluted rabbit anti *Brucella abortus* antiserum(25 μl) were mixed in the test plate and left to react at 4°C overnight. The agglutination pattern was an indicator of the reaction intensity.

RESULTS AND DISCUSSION

The main purpose of the work was to show that oxidised bacteria can serve as a useful particulate solid phase in agglutination immunoassays. Figure 1. shows the change of the optimal agglutination pattern with periodate con-

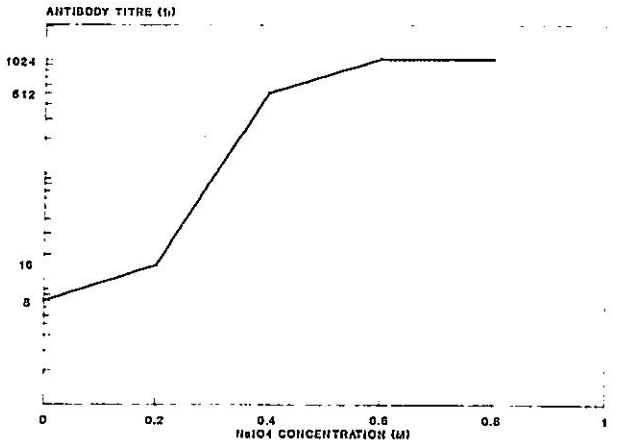

Figure 1. Changes in the greates antibody dilution required for visible agglutination with the concentration of $NaIO_4$ used for oxidation of the bacteria.

centration. hIgG in the concentration of 10.0 mg/ml was used for coupling, at 20° C for 18 h.

The results indicate that strong oxidation conditions (minimum of 0.4M $NaIO_4$) are necesary to oxidise the bacterial polysaccharides. The glyco component of the bacterial cell wall is more resistant to oxidative changes compared to free glycoproteins or polysaccharides.

An oxidation period of 2 h and 3 h was not sufficient to change the bacteria significantly (the amount of immobilized hIgG was very small), while oxidation for 18 h resulted in the formation of large aggregates of oxidised bacteria (detected both visually and microscopically). Oxidation for a period of 6 h made the bacteria very reactive towards hIgG and no agregates were seen under the microscope.

Figure 2. shows the agglutination pattern when different amounts of hIgG were offered to bacteria oxidised with 0.6M $NaIO_4$ for 6 h. The coupling time

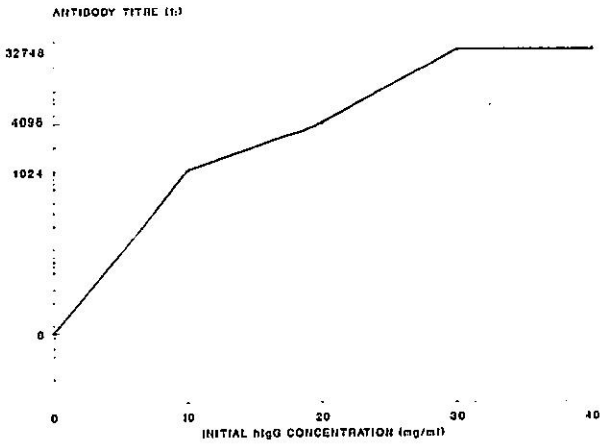

Figure 2. Changes in the greatest antibody dilution at which visible agglutination occured with the initial concentration of hIgG used to coat the bacteria.

was 18 h and the incubation temperature was 20° C. Up to the initial concentration of 10.0 mg/ml the hIgG agglutination was negligible, from 10.0 to 30.0 mg/ml the final hIgG dilution of antibody required for visible agglutination increased, while the concentration change from 30.0 to 40.0 mg/ml hIgG had no effect. Nonspecific agglutination with normal sheep serum appeared up to a 1:2 dilution of the initial serum.

The effect of the coupling period was investigated by measuring the agglutination pattern after coupling the bacteria (oxidised with 0.6M $NaIO_4$ for 6 h) with 30.0 mg/ml hIgG at 4° C and 20° C for 2 h, 3 h, 6 h and 18 h. The incubation conditions of 20° C and 18 h were necessary for significant coating to occur. All the results indicated that *Brucella abortus* could be coated with hIgG under quite severe conditions, but large quantities of hIgG were reguired for coating until saturation.

Another disadvantage of the described procedure was that a great amount of the bacteria sedimented at 300 x g in the form of aggregates. The working suspension contained 3.76 x 10^9 bacteria/5ml, which was 12.33% of the starting bacterial suspension.

We tried to overcome this problem by modifying the initial procedure (A) in two ways: one was to centrifuge the bacterial suspenson after the oxidation step (B) and other was to change the washing after hIgG coupling (C) and (D). The results of the microscopic pellet analysis for each procedure are presedented in Table 1.

Following the coating process (A), all inorganic crystals and most of the bacterial aggregates were sedimented at 30 x g. At 300 x g small bacterial aggregates and a few bacteria were brought down. After oxidation (B), the pellet collected at 30 xg contained only crystals, while that at 300 x g had very small crystals and a few bacteria. It follows from this analysis that the oxidation

process did not initiate bacterial cross-linkage, so that the aggregates formed only in the presence of hIgG. Only bacteria were detected in the sediment after the coating step. Using BSA (C) and TWEEN 20 (D) in the washing solution; i. e. changing the dielectric constant of the washing medium, hIgG coated bacteria were forced to orientate and behave differently than in pure PBS. That difference was directed towards the formation of smaller bacterial aggregates than those that resulted from the standard procedure (A).

Table 1. Microscopic pellet analysis

CENTRIFUGATION PROCEDURE	AFTER OXIDATION 30xg	AFTER OXIDATION 300xg	AFTER COATING WITH hIgG 30xg	AFTER COATING WITH hIgG 300xg
A	—	—	crystals and lagre bact. aggregates	small bact. aggregates and few bacteria
B	crystal agglomerates	crystals and few bacteria	large bact. aggregates	smal bact. aggregates and few bacteria
C	—	—	crystal aglomerates and smal bact. aggregates	crystals and few bacteria
D	—	—	crystal aglomerates and smal bact. aggregates	crystals and few bacteria

The quantitative effect of proces variation was determined by measuring the amount of bacteria remaining in the working suspension and by evaluating the applicability of each working suspension in the agglutination assay.

The amount of bacteria in the working suspension and the degree of utilisation of bacteria in each immobilisation procedure are given in Table 2.

Table 2. The amount of baccteria in working suspensions resulting from different coating procedures.
Starting suspension: 3.05×10^{10} bacteria (100%).

AMOUNT OF BACTERIA PROCEDURE	NUMBER OF BACTERIA IN 5 ml	PERCENTAGE OF THE INITAL NUMBER OF BACTERIA
A	3.76 x 109	12.33%
B	8.05 x 108	2.64%
C	6.48 x 108	2.11%
D	8.92 x 108	2.92%

Figure 3. shows the behaviour of each bacterial suspension in the agglutination assay.

The amount of bacteria in the working suspension obtained by the standard mode of immobilization was 12.33% of the initial amount. These bacteria exhibited nonspecific agglutination up to 1:2 dilution of normal sheep serum and specific agglutination up to 1:32768 dilution of sheep anti hIgG serum.

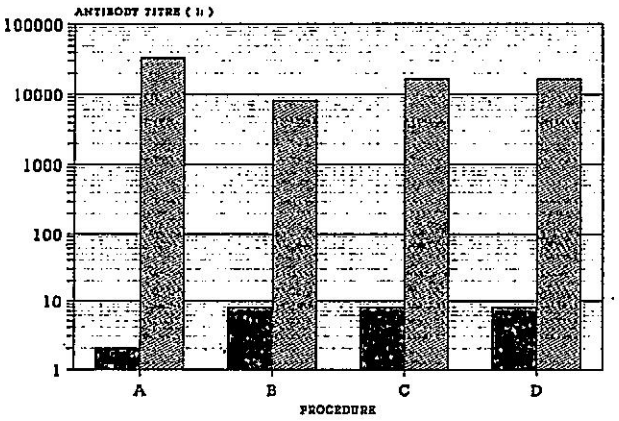

Figure 3. Nonspecific and specific agglutination of the hIgG coated bacteria obtained in a standard (A) and modified immobilization processes (B, C and D) with normal and anti-hIgG sheep serum. ▩ Nonspecific agglutination, ▨ Specific agglutination

The number of bacteria in working suspensions resulting from the modified immobilisation procedures were significantly lower than in the standard suspension, ranging from 2.11 to 2.92% of the initial number.

The nonspecific agglutination of all three nonstandard bacterial suspensions was up to 1:32 dilution of the antiserum. The increase in the length of the agglutination patern is probably due to the decrease in the concentration of hIgG coated bacteria in the reaction well.

Specific agglutination of the bacteria that were separated from inorganic crystals before coating with hIgG occurred up to the antiserum dilution of 1:8192. The shorter agglutination pattern suggested that oxidation of bacteria was not terminated after 6 h. Bacterial suspensions that contained BSA (C), or TWEEN 20 (D) showed the same intensity of specific agglutination, up to the antiserum dilution of 1:16384. Since decreasing the bacterial concentration increased the length of the agglutination pattern, these specific agglutinations were far weaker compared to the standard specific agglutination (A).

A possible explanation is that these hIgG coated bacteria, although they formed smaller aggregates, were surrounded by BSA or TWEEN 20 molecules which interupted immunological reactions.

Summing up our experience concerning the agregate analysis, we can conclude that the procedure initially applied for oxidation and hIgG immobilization, termed "procedure A", resulted in the bacterial suspension prepared under optimal conditions. The solutions for the problem of aggregates lies probably in sonification of the working suspension in order to disprese existing bacterial clumps.

The coanting efficiency was measured using ^{125}I-labeled hIgG as a tracer (1×10^6 cpm) in the solution of 30.0 mg/ml hIgG. The experimental conditions were those that had been found to be the best in previous test: 18 h and 20°

C. The washing procedure was the same as with unlabelled hIgG. After separating the aggregates at 300 x g, the radioactivity of aggregates and of the working bacterial suspension was measured.

We found that 1.82% of the initial hIgG was incorporated into the aggregates, i. e. 0.55 mg of the hIgG was immobilized on the bacteria that were sediment at 300 x g, while 4.48% of the initial hIgG was attached to the bacteria in the working suspension, i. e. 1.34 mg of the hIgG was bound on a form that was subjected to a further agglutination reaction.

The stability of the hIgG-bacteria link was investigated by repeating the agglutination assay at weekly intervals, for three months. As three was no change in the agglutination pattern during this period, hIgG coated bacteria could be stored (at +4° C) at least for the tested time and probably longer.

Finally, as strong oxidation conditions were applied, and as a significant amount of hIgG was immobilized on the bacterial surface, we wished to find out whether the antigenic determinants of *Brucella abourts* were affected by the coating procedure.

Agglutination test of the untreated and hIgG coated bacteria were carried out in a reaction with the standard rabbit anti *Brucella abortus* serum. In both cases bacteria agglutinated with the antiserum in same final dilution, which indicated that most, if not all, of the characteristic bacterial antigens were preserved and were not affected in terms of their immunoreactivity. Thus, *Brucella abortus* antigenic determinants responsible for the specific imunoreaction are not involved in the oxidation and the coating process.

Bacteria have not been widely used as a carrier and reports concerning this topic are not numerous. In one of them Messner et al. (1992) described the utilization of bacteria as carbohydrate hapten carriers.

Our work has confirmed that bacteria deserve more attention as a prosible particulate solid phase for the agglutination assay, since bacteria are easy and cheap to obtain, compared to some other solid phases, and they are very uniform in size.

This wotk was intended to explore the possibility of bacterial activition via surface oxidation. The results that we have obtained demonstrate that bacteria treated and coated with hIgG in the manner described, can serve as protein carrier in agglutination immunoreactions.

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BAKTERIJE KAO NOSAČI PROTEINA U TESTOVIMA AGLUTINACIJE

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SADRŽAJ

U radu se opisuje primena bakterija *Brucella abortus* kao čvrste faze za koju se mogu vezati proteini. Ugljenohidratne jedinice na površini bakterija oksidovane su sa $NaIO_4$ i za bakterije su kovalentno vezani molekuli IgG izdvojeni iz seruma ljudi. Rezultati aglutinacije su potvrdili da je ostvarena aktivacija bakterijske površine, kao i vezivanje IgG.